

Studies on Enzymes of the Capsaicin

Biosynthetic Pathway in

Capsicum frutescens.

by

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Susan S. Holland

Abbreviations

AOPP	Amino oxyphenyl propionic acid
ATP	adenosine 5'-triphosphate
BSA	Bovine serum albumin
butyl-PBD	2-(4'- <i>tert</i> -butylphenyl)-5-(4''-biphenyl)-1,3,4-oxadiazole
°C	degrees centigrade
<i>ca.</i>	approximately
Ca ²⁺	calcium ion
cat. no.	catalogue number
C ₄ -H	Cinnamate 4-hydroxylase
cm.	centimetre(s)
CPA	chlorophenoxyacetic acid
cpm.	counts per minute
cv.	cultivar
d.	day(s)
1D-	one dimensional
2D-	two dimensional
dpm.	decays per minute
2,4-D	2,4-dichlorophenoxyacetic acid
EDTA	ethylenediamine-tetra acetic acid
eg.	for example
<i>et al.</i>	<i>et alia</i>
f.wt.	fresh weight
g.	gram(s)
xg.	x gravitational force
h.	hour(s)
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
ie.	that is
l.	litre(s)
m.	metre(s), milli-
μ.	micro-
M.	molar
max.	maximum
mCi.	millicurie(s)
mg.	milligram(s)
min.	minute(s)
ml.	millilitre(s)
mM	millimolar
mRNA	messenger ribonucleic acid
mol.	mole(s)
MS	Murashige and Skoog
μC.	microcurie(s)
μl.	microlitre(s)
n.	nano-
N ₂	nitrogen
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
nd.	not detected
nm.	nanometre(s)

no.	number
PAL	Phenylalanine ammonia-lyase
%	percent
pH	negative log of the hydrogen ion concentration
PMSF	phenylmethyl sulphonyl fluoride
ppi.	pores per inch
POPOP	1,4-Di-2-(5-phenyloxazolyl)-benzene
PPO	2,5-Diphenyloxazole
psi.	pounds per square inch
PVP	polyvinylpyrrolidone
Rf.	relative front
rpm.	revolutions per minute
s.e.	standard error
sec.	second(s)
SH	Schenk and Hildebrandt
TLC	thin layer chromatography
1D-TLC	one-dimensional thin layer chromatography
2D-TLC	two-dimensional thin layer chromatography
UV	ultraviolet
v/v	volume per volume (as percentage)
w/v	weight per volume (as percentage)
>/<	greater than/less than

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Abstract

The aim of this project was to investigate the regulation of capsaicin biosynthesis in *Capsicum frutescens* (*C. frutescens*). Selected enzymes in the pathway were studied and particular attention was paid to those at the top of the biosynthetic pathway and also the terminal reaction sequence catalysed by the capsaicinoid synthesizing complex (referred to as capsaicin synthase).

Capsaicinoids and intermediates of the phenylpropanoid pathway were extracted from plants and cell cultures using techniques which were optimized. In the majority of cases, high performance liquid chromatography was used for the separation and analysis of compounds. An assay procedure was developed for measuring capsaicin synthase activity *in vitro* utilizing the substrates [³H]vanillylamine and 8-methyl-6-nonanoic acid and the cofactors ATP and Coenzyme A. The catalytic activities of phenylalanine ammonia-lyase (PAL) and cinnamate 4-hydroxylase (C,4-H) were extracted and assayed according to methods reported in the literature.

The specific activities of PAL and C,4-H did not increase prior to the onset of capsaicin accumulation. Neither were differences in the specific activities of these two enzymes observed between cells which incorporated [¹⁴C] phenylalanine into capsaicin and those which did not.

Capsaicin was shown to start accumulating in the fruits of *C. frutescens* ca. 18d. following fruit initiation. However, capsaicin synthase activity was shown to be present in fruits ca. 8d. prior to the onset of capsaicin accumulation.

Measurable capsaicin synthase activity was extracted from suspended cells of the chilli pepper which were approaching the stationary phase of the growth curve. No extractable capsaicin synthase activity was detected in suspended cells at 0, 7, 14d. following subculture. The activity of capsaicin synthase was shown to be increased in suspended cells which had been subjected to a nutrient stress regime. Elicitation with a fungal spore preparation was also shown to increase the amount of extractable capsaicin synthase activity.

Since capsaicin synthase activity appears prior to the onset of capsaicin accumulation, it seems likely that substrate availability may play a part in the regulation of capsaicin synthesis. The possible regulation of the capsaicin biosynthetic pathway is discussed.

Chapter 1

Introduction

Capsicum and the Capsaicinoids

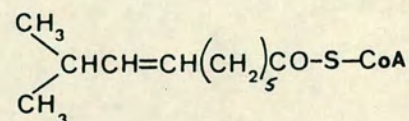
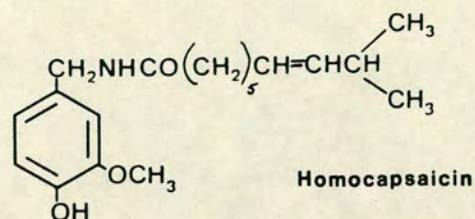
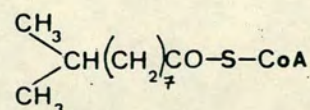
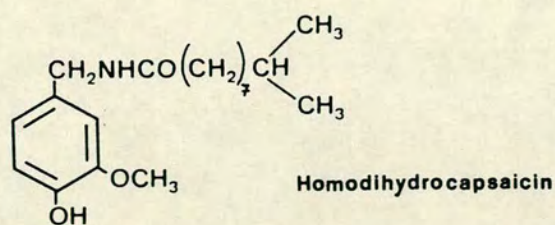
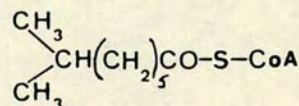
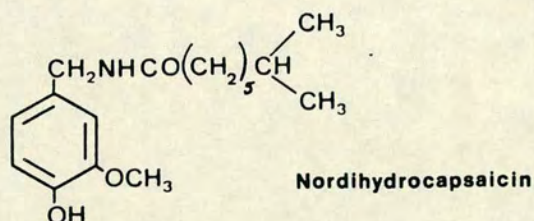
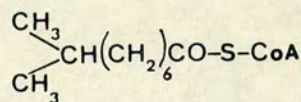
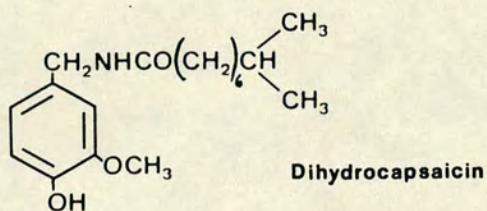
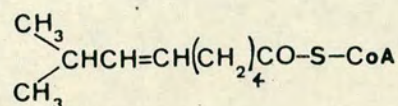
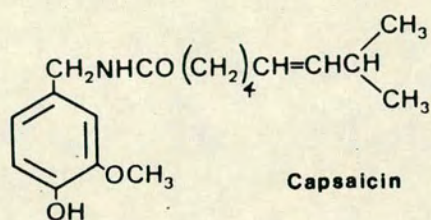
The genus *Capsicum* contains 20–30 species of domesticated and wild chilli peppers and around the same number of species of bell pepper (Pickersgill, 1987). The wild forms of the species *Capsicum frutescens* are distributed through much of south and middle America (Pickersgill *et al.*, 1979) and include some of the hottest peppers known. The secondary compounds responsible for the hot taste in the fruit are the capsaicinoids of which capsaicin is the major component (Purseglove *et al.*, 1968). Capsaicin is a moderately valuable plant product which is used in the food flavouring and pharmaceutical industries. Obtained in the crude form as an oleoresin capsaicin can cost up to £450 per kilogram (Yeoman *et al.*, 1989), whereas in the pure form it can be obtained from the Sigma chemical Co at a cost of £84 per gram (Sigma Ltd. 1989). In addition to capsaicin, four other capsaicinoids have been isolated and identified (Masada *et al.*, 1971). The structures of all five naturally occurring capsaicinoids can be seen in Fig. 1.1. All of these capsaicinoids are believed to be formed by the condensation of vanillylamine with one of a number of acyl groups. The resulting composition of the various vanillyl amides in fruits of *C. frutescens* is usually *ca.* capsaicin (69%), dihydrocapsaicin (22%), nordihydrocapsaicin (7%), homocapsaicin (1%) and homodihydrocapsaicin (1%) (Leete and Loudon, 1968; Bennett and Kirby, 1968). Since capsaicin is a commercially valuable secondary metabolite, the study of capsaicin synthesis and accumulation is an important part of any investigation where increased yields of this product are required.

Fig. 1.1

Structures of the five capsaicinoids which occur naturally, along with the corresponding activated acyl groups which condense with vanillylamine to form the capsaicinoids.

CAPSAICINOID

ACTIVATED ACYL DERIVATIVE



Primary and Secondary Metabolism

Primary metabolism has been defined as:

"processes that produce essential cellular constituents, that enjoy universal roles in cellular metabolism (eg. amino acids, etc)" (Jensen, 1986).

and secondary metabolism has been defined as:

"processes that lead to the synthesis of a diverse group of compounds accumulated by plant tissues, which although not essential to the life of the cell in which they are formed may give the organism which contains such cells a greater degree of fitness for survival" (Hall, 1984).

While this definition between primary and secondary metabolism is useful, the boundary between the two areas is nevertheless imprecise (see Haslam, 1986). However, with respect to capsaicin synthesis, this distinction between primary metabolism and secondary metabolism is useful since the balance of these two will affect the availability of substrate for capsaicin synthesis. For the purposes of the present investigation, secondary metabolism will be defined as all steps following PAL, although it is recognised that several steps after PAL (eg. conversion of cinnamate and ferulate into cell wall derivatives) should be classified as primary metabolism.

The Proposed Biosynthetic Pathway of Capsaicin Synthesis

The biochemical pathway leading to the formation of capsaicin has not yet been fully elucidated, but the most likely steps are shown in Fig. 1.2. The pathway has two branches: the phenylpropanoid component is derived from phenylalanine and the acyl moiety from valine. Thus radioactively labelled phenylalanine, cinnamate derivatives and vanillylamine are incorporated *in vivo* into the ring structure of capsaicin (Bennet and Kirby, 1968) and valine is incorporated into the acyl moiety (Leete and Loudon, 1968). The enzymes which catalyse the early steps of the phenylpropanoid reaction sequence are well characterized. Phenylalanine ammonia-lyase (PAL) [E.C. 4.3.1.5.] catalyses the elimination of ammonia from L-phenylalanine to form *trans*-cinnamic acid, this being the first committed step of phenylpropanoid biosynthesis in higher plants (Hanson and Havir, 1981). PAL was discovered by Koukol and Conn (1961) and since then it is probably the most studied enzyme concerned with secondary metabolism in plants (Camm and Towers, 1977). This is because a number of studies have suggested that PAL catalyses the rate-determining step between phenylalanine and phenylpropanoid metabolism (Camm and Towers, 1973; Hahlbrock and Grisebach, 1979). Consistent with this idea are the position of PAL at the beginning of the pathway and the ready inducibility of increased PAL activity under conditions which increase phenylpropanoid metabolite levels (Dixon and Lamb, 1979; Betz *et al.*, 1978; for complete review, see Jones, 1984).

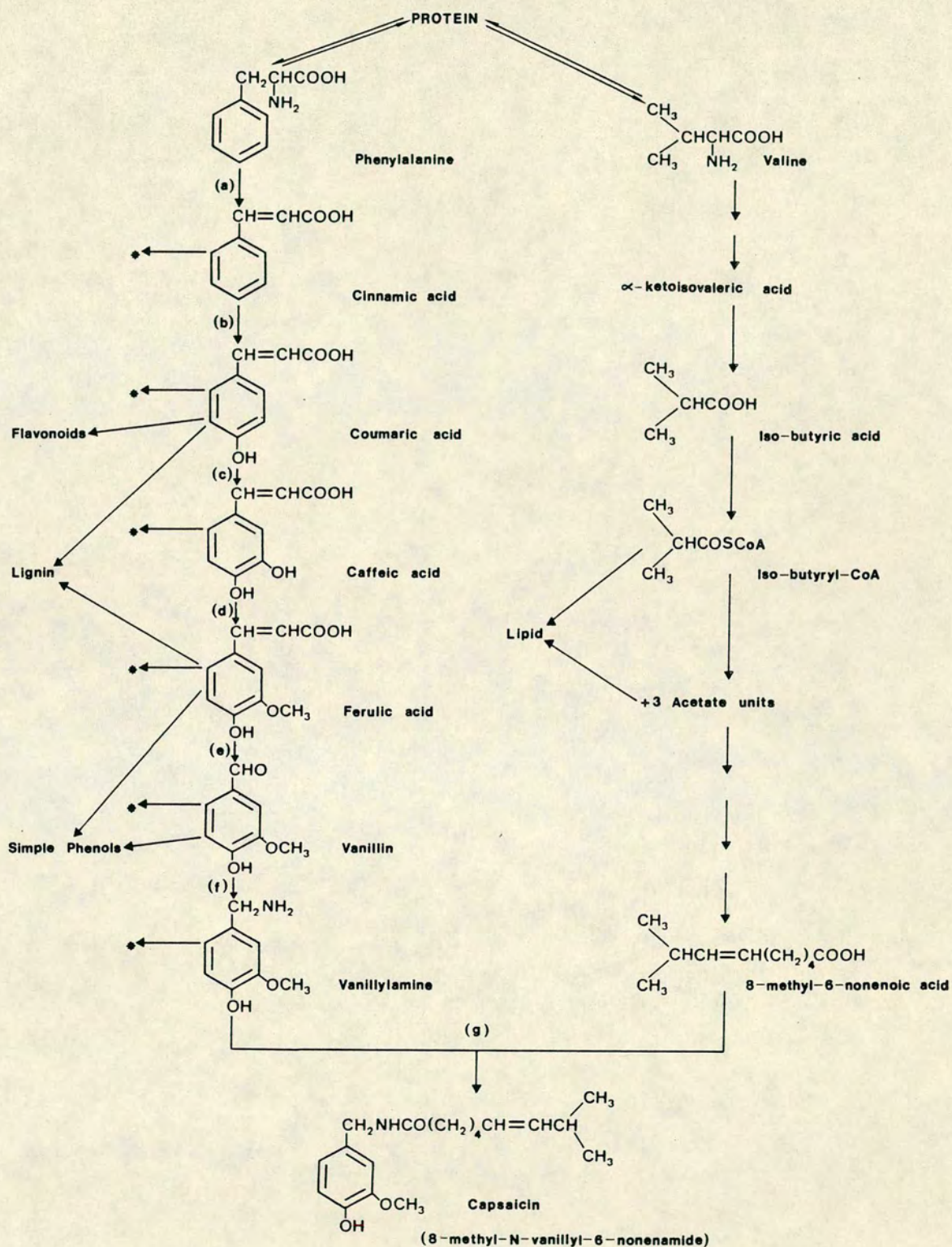
Fig. 1.2.

The Proposed Biosynthetic Pathway of Capsaicin

Enzymes:

- (a) = Phenylalanine ammonia-lyase (PAL)
- (b) = Cinnamate 4-hydroxylase (C,4-H)
- (c) = *p*-Coumarate 3-monooxygenase
- (d) = not characterized
- (e) = not characterized
- (f) = not characterized
- (g) = Capsaicin synthase

* = esterified/conjugated forms of the acid.



Cinnamate 4-hydroxylase (C,4-H) [E.C. 1.14.13.11.] catalyses the second step in the phenylpropanoid pathway where cinnamic acid is converted to *p*-coumaric acid in the presence of NADPH (Russell and Conn, 1967; Stafford, 1969; Potts *et al.*, 1974; Lamb and Rubery, 1975). This enzyme has also been extensively studied, particularly since the importance of cinnamic acid and cinnamoyl-CoA derivatives as central intermediates in the biosynthesis of a wide range of plant phenolics has been recognized (Amrhein and Zenk, 1977; Zenk, 1977). The enzyme which catalyses the conversion of *p*-coumaric acid to caffeic acid (*p*-coumarate 3-monooxygenase; E.C. 1.14.17.2.) has been characterized in *Beta vulgaris* (Vaughan and Butt, 1969) and other work has demonstrated the importance of this enzyme in the metabolism of phenolic substances in plants (Brown, 1966; Neish, 1964; Steck, 1968). A number of reports have shown that C,4-H activity can be coordinately induced with PAL (Tanaka *et al.*, 1974) and there is a small amount of evidence supporting the view that PAL and C,4-H may be present as membrane-bound multienzyme complexes (Czichi and Kindl, 1977; Hrazdina and Wagner, 1985). It is likely that interconversion of the cinnamic acid derivatives occurs via mechanisms involving Coenzyme A. Accordingly it has been shown that the synthesis of cinnamoyl-CoA (Walton and Butt, 1971), *p*-coumaroyl-CoA (Hahlbrock and Grisebach, 1970; Lindl *et al.*, 1973) and feruloyl-CoA (Gross *et al.*, 1973) occur in higher plants and there are a number of reports describing the isolation and partial characterization of some of the CoA:ligase enzymes (Gross and Zenk, 1974 and refs therein). It is therefore unlikely that the proposed biosynthetic pathway in Fig.1.2. is complete since the intermediates of the capsaicin pathway may be converted as Coenzyme A derivatives as well as in the free form. The scheme merely serves as a guide and attempts to summarize, on the basis of evidence available, what are the likely steps.

Virtually nothing is known about the enzymes towards the end of the pathway in particular those which catalyse the steps between ferulic acid and capsaicin. There are, however, three articles which report the presence of a capsaicin synthesizing enzyme complex in fruits of *C.annuum* var. *annuum* cv. Karayatsubusa (Fujiwake *et al.* 1980a,b and 1982) but there is no indication in these papers as to how many enzymes are involved or whether the activity is correlated with the onset of capsaicin synthesis and accumulation. Also, little is known of the enzymes associated with the acyl branch of the pathway in peppers although valine and leucine have been shown to act as precursors of

capsaicin (Leete and Loudon, 1968). However, this does not confirm that the acyl moiety is always derived from these two molecules *in vivo* and it is possible that the formation of 8-methyl-6-nonenic acid occurs via a reaction sequence not dissimilar to normal fatty acid biosynthesis, where acetate units might be sequentially added to the "iso-unit" to form the range of branched chain acyl groups shown in Fig. 1.1. Work to date in this laboratory has concentrated on the phenylpropanoid branch of the pathway as the exogenous supply of precursors to this side of the pathway has been shown to enhance capsaicin yield (Lindsey *et al.*, 1983).

Attempts to Increase the Yield of Capsaicin by Cell Cultures

The production of secondary metabolites by plant cell cultures has been under active investigation for a number of years and a large variety of compounds have been produced in a wide range of species (Dougall, 1980. Cvikrova *et al.*, 1988 and Ozeki and Komamine, 1985). The advantage of studying secondary metabolite production in cell cultures is that the cells are removed from the complex environment of the plant and placed in a controllable, defined environment which allows the effect of individual treatments to be examined. In addition, there are several advantages associated with the production of useful compounds for commercial purposes by cell cultures. These include: freedom from climatic restrictions and diseases, reduction in the requirement for agricultural land, together with the assurance of a qualitatively and quantitatively consistent product. However, as far as secondary metabolites are concerned, plants are generally more efficient at accumulating these substances than the cultures derived from them (Fowler, 1983) and only in a minority of cases do the cultures accumulate secondary products more efficiently than the plant (Fowler, 1986). Despite a long and intensive period of research only two compounds, shikonin and berberine, obtained from cultures of *Lithospermum erythrorhizon* and *Coptis japonica* respectively are being produced commercially (Mitsui Petrochemical Industries, Tokyo).

Generally, plant cell cultures which are fast growing seldom accumulate secondary metabolites at levels which occur in the intact plant and cultures of *C.frutescens* growing on standard growth medium do not produce detectable levels of capsaicin. There is good evidence to show that accumulation is usually related to a slow or zero growth rate, aggregation and/or the presence of recognisable plant organs (Yeoman *et al.*, 1980). It is also thought that at some stage in the development of a culture there is a switch in cell biochemistry away from primary towards secondary metabolism.

Empirical studies have been employed extensively to enhance capsaicin yield in cultures of *C.frutescens*. Indeed, techniques involving manipulation of the chemical environment eg. nutrient limitation (Yeoman *et al.*, 1980; Lindsey, 1985) precursor feeding (Lindsey and Yeoman, 1983) and the manipulation of the physical environment eg. cell immobilization (Lindsey *et al.*, 1983) have all been reported to increase the yield of capsaicin. More recently, the use of fungal and abiotic elicitors has also been shown to enhance capsaicin accumulation (Holden *et al.*, 1988a,b). Whilst these techniques have been successful in enhancing yield (Lindsey, 1985), results obtained from these investigations have given little indication as to how the metabolism of these cells is altered by such manipulations. The changes in metabolism which facilitate capsaicin production may be related to substrate supply or to the level of activity of a few key enzymes or both. Therefore what is required is a full characterization of the enzymes involved in the biosynthetic sequence in order to obtain an understanding of how the pathway is regulated.

Studying Metabolic Regulation

A biochemical pathway consists of a series of enzyme-catalysed reactions that convert substrate(s) into product(s). There are essentially two major objectives in the experimental analysis of a given biochemical pathway; the first is the identification of the chemical sequence and the stoichiometry of each reaction. This may be achieved by the application of various techniques including: identification of intermediates which accumulate upon addition of highly selective inhibitors, addition of possible intermediates of the pathway (since their conversion to the product confirms this role) and the use of radioactively labelled substrates and study of the distribution of label in the intermediates and product. This approach also involves the extraction of each enzyme in the sequence, its purification and investigation of its specificity, kinetics, mechanism and inhibition. The results of these investigations are then collated into a metabolic "map" which essentially describes the sequence of reactions. It does not however explain the process known as metabolic regulation which is the interaction and control of all the individual steps. The second major part in an investigation of this type is therefore the identification of the mechanisms by which the pathway is regulated. This involves the identification of the regulatory enzymes in the pathway and defining their response to specific modulating metabolites. Although a vast amount of work has been published since the 1950s on the regulation of various metabolic sequences ranging from primary metabolism eg. glycolysis (Williamson, 1965; Newsholme *et al.*, 1977; Bosca and Corredor, 1984) and gluconeogenesis (Newsholme and Gevers, 1967) to secondary metabolic processes eg. phenylpropanoid metabolism (Margna, 1977) there is still debate about the concept of rate-limiting enzymes (Kascer and Burns, 1973; Kascer and Porteous, 1987). In many of these reports it is recognized that complicated reaction networks must be rigorously controlled to avoid wasteful dissipation of energy and carbon units, and yet, metabolic regulation must remain flexible because the external environment of the cells is not constant. Metabolic regulation must not therefore be viewed as an "on/off" switch, but more as a sensitive detection and adjustment system that allows for second-to-second changes in the requirements of cells.

A number of research workers have proposed ways in which the phenylpropanoid pathway might be regulated. The activities of constituent enzymes obviously play an important role (Hahlbrock *et al.*, 1976; Ebel *et al.*,

1974) as might the structural organisation of those enzymes (Hrazdina and Wagner, 1985; Czichi and Kindl, 1977; Wagner and Hrazdina, 1984). In addition the availability of substrates will dictate whether or not phenylpropanoids are synthesized (Margna, 1977; da Cunha, 1987). Nevertheless, it is likely that identification of key enzymes will lead to a greater understanding of how capsaicin synthesis is regulated and facilitate manipulation of the balance of metabolism in favour of capsaicin formation and accumulation in cell cultures.

Possible Regulatory Steps in the Capsaicin Biosynthetic Pathway

Previous work has shown that although incorporation of [^{14}C]phenylalanine into capsaicin is increased when pepper cells are immobilized, the specific activities of PAL and C₄-H are lower in immobilized cells than in suspended cells. This suggests that the increased flow of metabolites through the pathway resulting in increased capsaicin synthesis in immobilized cells, was not due to a rise in the specific activities of either of these two enzymes (Holden *et al.*, 1987a). However, fungal elicitation which leads to an increase in PAL activity also results in increased capsaicin synthesis (Holden *et al.*, 1988a), presumably because of an increase in the flow of metabolites down the phenylpropanoid pathway. As the intermediates of the pathway from cinnamate to ferulate are likely to be present in all plant tissues because they are required for cell wall synthesis, it is reasonable to assume that the enzymes necessary to convert phenylalanine to vanillin are also present in the vegetative tissues of the chilli pepper. However, only the fruit can produce capsaicin and one interpretation of this situation is that only the fruit has the necessary enzyme(s) to complete the reaction sequence from vanillin to capsaicin. Alternatively, it may be that the supply of substrate to the phenylpropanoid and/or acyl branch of the pathway regulates the synthesis and accumulation of capsaicin. Both of these possibilities were studied in this investigation.

Aims and Objectives

The aim of this project was to investigate the regulation of capsaicin synthesis through a study of selected enzymes in the capsaicin biosynthetic pathway using fruits and cell cultures of *C.frutescens*. The following objectives were identified:

- 1) To confirm earlier findings regarding the likely position of the regulatory step in the capsaicin synthetic pathway.
- 2) To develop a reliable and sensitive *in vitro* extraction and assay procedure for capsaicin synthase.
- 3) To attempt to correlate capsaicin synthase activity with the onset of capsaicin accumulation in fruits and cultured cells of *C.frutescens*.
- 4) To increase capsaicin synthase activity in cultured cells of *C.frutescens* using selected nutrient and stress regimes.
- 5) To determine the kinetics of the capsaicin synthase reaction following extraction of the enzyme from cultured cells of the chilli pepper.

Chapter 2

Materials and Methods

2.1. Plant Material and Growth Conditions.

Plants of *Capsicum frutescens* cv. annum were grown from seed (McNair, Edinburgh) in 7.5cm. pots in damp Levington's compost (Fison's Ltd, U.K.) under strictly controlled growth room conditions; $25 \pm 1^\circ\text{C}$, illuminance $135 \mu\text{mol.m}^{-2}\text{sec}^{-1}$ (Thorn warmwhite fluorescent, Phillips tungsten), 16h. day. Watering was carried out as required to keep the compost moist. After 3 weeks the seedlings were transplanted to 15cm. pots in which the plants reached flowering (ca. 6 weeks after germination). Individual flowers were numbered and the date of fertilization recorded, this facilitated the selection of fruits of a similar age for use in experiments (Fig. 2.1.).

2.2. Tissue and Cell Culture.

2.2.1. Preparation of Culture Media.

2.2.1.1 Schenk and Hildebrandt medium and Murashige and Skoog medium.

Two types of basal media were used for *in vitro* studies. Both Schenk and Hildebrandt (SH) medium (Schenk and Hildebrandt, 1972) and Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) were prepared from powdered mixtures supplied by Imperial Laboratories (Cat no. 9-650-50), Twyford, U.K., and Flow Laboratories Ltd (Cat no. 26-100-24), Irvine, Scotland respectively. The media were prepared by dissolving the appropriate amount of powder (4.46 g.l^{-1} SH, and 4.71 g.l^{-1} MS) in distilled water along with 30 g.l^{-1} of sucrose as a carbon source. The constituents of each of the media can be found in Table 2.2.1. The appropriate growth regulators and other components were added prior to adjustment of the pH to 5.8 using M. potassium hydroxide (BDH Ltd., Poole, Dorset). The medium was then made up to the required volume with distilled water.

In addition to the above constituents, solid media contained 10 g.l^{-1} agar (Oxoid No.1. Oxoid Ltd., Basingstoke, Hampshire) added after adjustment of the pH.

Fig. 2.1.

Plant of *C.frutescens* ca. 10 weeks showing tagged fruit which was marked at fruit initiation.



2.2.1.2. Media lacking in nitrogen.

MS and SH media lacking in nitrogen were used in experiments described in section 3. The constituents of these media are listed in Table 2.2.1. they were dissolved in distilled water immediately prior to use. Growth regulators were added at the same concentration as for normal growth media (section 2.2.1.7). For some stress treatments sucrose was excluded from the medium. The pH was adjusted to 5.8 using M.potassium hydroxide before the medium was made up to the required volume with distilled water. No adjustment to the potassium level was made to account for the reduced potassium level in media deficient in nitrogen since it is extremely difficult to alter the balance of one ion and not affect the balance of another. Given that potassium was supplied in the form of KH_2PO_4 observed differences between treatments were likely to be due to nitrogen deficiency rather than potassium deficiency.

Table 2.2.1.

The compositions of Schenk and Hildebrandt medium with nitrogen (SH), without nitrogen (SH-N) and Murashige and Skoog medium with nitrogen (MS), without nitrogen (MS-N) are as follows:

Constituents	Concentration in Media (mg.l. ⁻¹)			
	(SH)	(MS)	(SH-N)	(MS-N)
CaCl ₂ .2H ₂ O	200	440	200	440
CoCl ₂ .6H ₂ O	0.1	0.025	0.1	0.025
CuSO ₄ .5H ₂ O	0.2	0.025	0.2	0.025
FeNaEDTA	-	36.7	-	36.7
FeSO ₄ .7H ₂ O	15	-	15	-
(Na ₂)EDTA	20	-	20	-
H ₃ BO ₃	5.0	6.2	5.0	6.2
KH ₂ PO ₄	170	170	170	170
KI	1.0	0.83	1.0	0.83
KNO ₃	2500	1900	-	-
MgSO ₄ .7H ₂ O	400	370	400	370
MnSO ₄ .4H ₂ O	10	22.3	10	22.3
NaMoO ₄ .2H ₂ O	0.1	0.25	0.1	0.25
NH ₄ H ₂ PO ₄	300	-	-	-
NH ₄ NO ₃	-	1650	-	-
ZnSO ₄ .7H ₂ O	1.0	8.6	1.0	8.6
<i>myo</i> inositol	1000	100	1000	100
nicotinic acid	5.0	0.5	5.0	0.5
thiamine HCl	5.0	0.1	5.0	0.1
pyridoxine HCl	0.5	0.5	0.5	0.5
glycine	-	2.0	-	2.0
<i>p</i> chloro-phenoxyacetic acid [CPA]	2.0	2.0	2.0	2.0
2,4-dichloro-phenoxyacetic acid [2,4-D]	0.5	0.5	0.5	0.5
kinetin	0.1	0.1	0.1	0.1
sucrose	30,000	30,000	30,000	30,000
pH	5.8	5.8	5.8	5.8

2.2.2. Sterilization Techniques.

2.2.2.1. Sterilization by heat.

All glassware, instruments, pretreated polyurethane foam blocks, distilled water and nutrient media which did not contain heat-labile compounds were sterilized by autoclaving at 121°C for 20min. at a steam pressure of 15 psi.

2.2.2.2. Sterilization with hypochlorite.

All plant material used for culture initiation was surface sterilized before use.

Seeds were subjected to a rapid pre-sterilization in 95% (v/v) ethanol in distilled water for 10sec. and then immersed for 30min. in 10% (v/v) sodium hypochlorite (1.5% available chlorine) in distilled water. The seeds were then removed aseptically and washed in sterile distilled water (3 times) before use.

Small segments (max. length 2cm.) of actively-growing stem tissue were washed in distilled water and the cut ends sealed with wax. The segments were subjected to a rapid pre-sterilization in 70% (v/v) ethanol in distilled water for 10sec. and then immersed for 30min. in 10% (v/v) sodium hypochlorite in distilled water. The stem segments were then washed as for the seeds.

2.2.2.3. Sterilization with ethanol.

All cell culture manipulations were carried out on the bench of a laminar flow cabinet over which a continuous stream of sterile air was passed. The work surfaces were swabbed and sprayed with absolute ethanol before and after culture manipulations. Instruments were stored in absolute ethanol and flamed immediately prior to use to remove surface ethanol.

2.2.2.4. Sterilization by filtration.

For feeding experiments with radioactively labelled precursors, solutions were filter sterilized using preautoclaved, 1ml. filter units containing a nylon-66 filter (0.22 μ m. pore size) (Anachem, Luton, U.K.).

2.2.3. Initiation and Maintenance of Cultures.

2.2.3.1. Culture conditions.

Unless otherwise stated in the text all cell cultures were grown under the following conditions:

Temperature	$25 \pm 2^{\circ}\text{C}$.
Photon flux density	$25 \mu\text{mol.m}^{-2}\text{sec}^{-1}$
Light source	Compton warmwhite fluorescent.
Liquid culture agitation	Continuous rotation in a horizontal plane, 98rpm., 0.8cm. amplitude.

2.2.3.2. Callus culture initiation, *C. frutescens*.

Callus cultures of *Capsicum frutescens* were grown in 9cm. polystyrene Petri-dishes (Sterilin Ltd, Hounslow, U.K.) containing 20–25ml. solid medium. The Petri-dishes were sealed with parafilm (American Can Company, Greenwich, U.K.) to exclude microorganisms and to prevent desiccation.

Sterilized seeds were plated onto SH or MS medium containing 2mg.l^{-1} CPA., 0.5mg.l^{-1} 2,4-D. and 0.1mg.l^{-1} kinetin. The hypocotyls from 3 week old seedlings of *C.frutescens* were removed aseptically and plated onto fresh medium. After a further 2 weeks, pale yellow, friable callus had formed on the cut ends of the hypocotyls.

2.2.3.3. Suspension culture initiation, *C. frutescens*.

Unless otherwise stated in the text, cell suspension cultures of *C. frutescens* were grown in 250ml. conical (Erlenmeyer) flasks, containing 50ml. liquid medium covered with a double layer of aluminium foil. The cultures were initiated by transferring 1–2g. (f.wt.) of friable callus to liquid medium in a conical flask which was then placed on an orbital shaker (section 2.2.3.1).

For less friable callus cultures (typically those initiated on MS medium), suspension cultures were initiated by separating out lumps of callus in a sterile Petri-dish with a sieve spoon after which the cells were transferred to liquid medium as previously described.

2.2.3.4. Maintenance of callus and suspension cultures, *C. frutescens*.

Stock cultures were maintained on both SH and MS media as callus and suspension cultures.

Callus cultures were subcultured every 2 weeks onto fresh solid media. Sterile forceps were used to transfer the callus. Cell suspension cultures of *C. frutescens* grew in aggregated clusters. A sterile sieve spoon was used to transfer 1-2g. of cells into 50ml. of fresh liquid media. The time interval between subcultures varied according to the growth rate of the cultures, but was usually 2 weeks.

2.2.4. Immobilization of Suspended Cells.

Cell suspension cultures of *C. frutescens* were immobilized in blocks of polyurethane foam as described by Lindsey *et al.* (1983). The polyether-type polyurethane foam (Declon Ltd., Corby, U.K.) was cut into 1.0 cm³ blocks which were then pretreated before use in the cell suspension culture system. The pore size of the foam was 40 pores per inch (ppi).

2.2.4.1. Pretreatment of the foam blocks.

The foam blocks were boiled for one hour in a large volume of distilled water and rinsed first with ethanol for 15 min. and then 3 times in distilled water to remove any toxic substances. The number of foam blocks required for each culture flask (usually 5) was then placed in a 50 ml. boiling tube which was then covered with a double layer of foil. The blocks were heat sterilized in these tubes as described in section 2.2.2.1.

2.2.4.2. The immobilization procedure.

Sterile blocks (usually 5) were added to a freshly subcultured suspension culture. The flask was then agitated vigorously by hand in order to submerge the foam blocks and placed on an orbital shaker (section 2.2.3.1). During the following two weeks, cells became entrapped then grew and divided within the foam matrix. At 4 weeks the blocks were transferred to fresh medium. Once densely packed with cells, the blocks were used in subsequent investigations.

2.3. Measurement of Culture Growth and Viability.

2.3.1. Determination of Fresh Weight.

The fresh weight of the suspended cells was determined after they had been vacuum filtered through damp Whatman 1. filter paper for 5 min. (Whatman International Ltd, Maidstone, U.K.). This procedure was standardized to ensure consistent separation of cells from their bathing medium.

2.3.2. Determination of Dry Weight.

A known fresh weight of cells was washed thoroughly with distilled water to remove surface sucrose and dried overnight (*ca.* 16h.) in a hot air oven at 60°C. The dry weight was determined after the sample had cooled to room temperature in a desiccator.

2.3.3. Determination of the Cell Population Density in Suspension Cultures.

The cell population density of liquid cultures was estimated with a Hawksley crystalite haemocytometer (grid vol 1.8 μ l).

Due to the nature of cell suspension cultures of *C. frutescens*, an initial digestion was necessary to loosen the cells from one another. To a known fresh weight of cells (*ca.* 0.4g) was added 5ml. of 5% chromic acid solution (w/v chromium trioxide in distilled water). This mixture was then left at room temperature for 24h. after which time it was homogenized by pumping the solution in and out of a Pasteur pipette 20 times. Sufficient distilled water was then added to give a cell density of 150–200 cells grid⁻¹. Ten grids were then counted, the mean of which was used to estimate the density of the original cell population.

2.3.4. Determination of Cell Viability

The method of Widholm (1972) was used which is based on the ability of living cells to cleave fluorescein diacetate molecules to produce free fluorescein which is detectable by its bright green/yellow fluorescence in ultraviolet light.

A stock solution of fluorescein diacetate (Sigma) was prepared by dissolving 25mg. in 5ml. of acetone. Immediately prior to analysis the stock solution was added dropwise to 5ml. of culture medium until a slight turbidity developed. One drop of this mixture was then mixed with one drop of cell suspension culture on a microscope slide and covered with a glass coverslip. After 10min. the cells were observed under visible and ultraviolet light. The proportion of cells with fluorescent protoplasm was determined in a random sample of *ca.* 500 cells.

2.4. Radiolabelling of plant material *in vivo*.

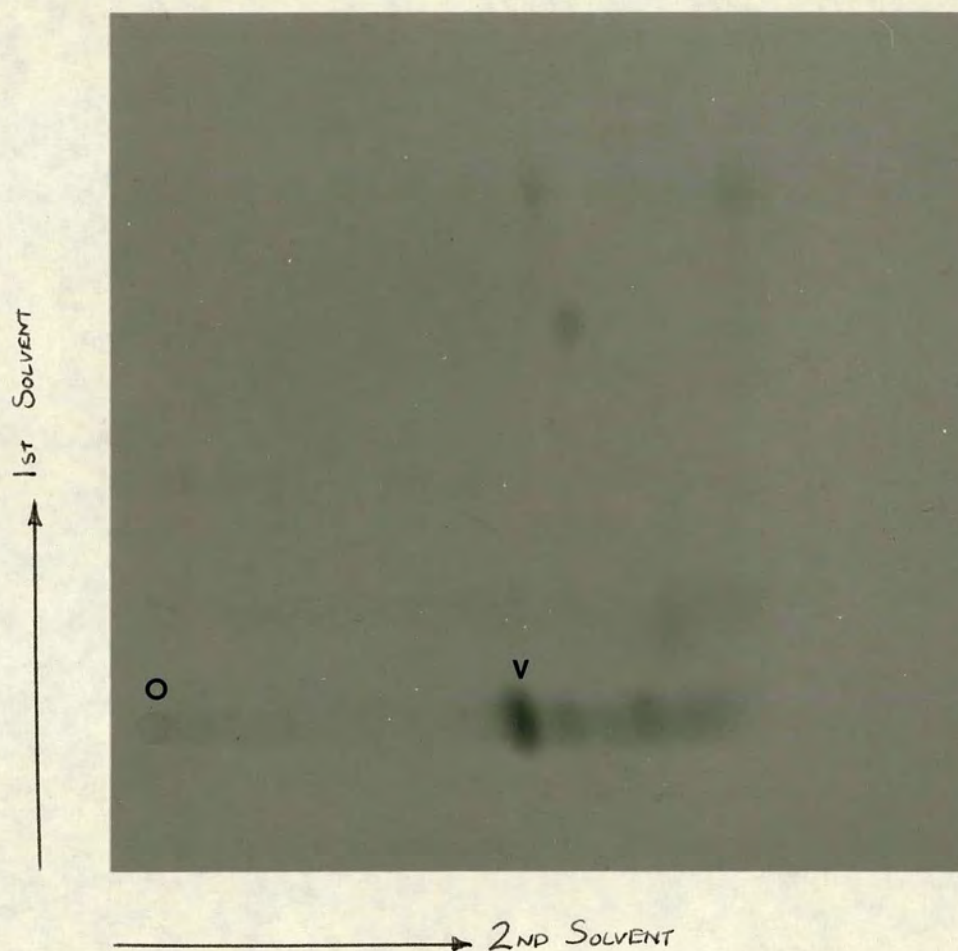
2.4.1. Purification of [³H]Vanillylamine.

Vanillylamine hydrochloride (100mg) (Aldrich Chemical Co.) was purified to 99.9% purity using preparative high performance liquid chromatography and sent to Amersham International (Amersham, U.K.) to be radio-labelled. The vanillylamine was tritiated using the catalytic exchange technique in solution with tritium gas. The product was diluted from 25ml. to 110ml. and stored as recommended by Amersham International at -20°C. The total activity supplied was 500mCi. and the specific activity was calculated to be approximately 1.5 Ci. mmol.⁻¹ (on receipt). Over a period of several months self-radiolysis of the product occurred and a complex mixture of labelled compounds resulted. It was therefore necessary to purify the stock solution immediately prior to experimental use.

The [³H]vanillylamine was purified routinely from the stock solution using 2D-thin layer chromatography (described in section 2.6.2.3). This system was found to separate all the major labelled contaminants from the [³H]vanillylamine (see Fig 2.4.1.). Following exposure of the plate to film (Hyperfilm-MP, Amersham, U.K.) overnight at -40°C (see section 2.6.2.4), the [³H]vanillylamine was located, the spot scraped off the plate and the compound extracted into an appropriate buffer (usually 1ml. of 0.2M, Tris-HCl pH 9.5). The mixture was vortexed for several seconds and then filtered to remove the silica gel particles (see section 2.2.2.4). The filtrate was then sealed in a glass vial with a crimp top and used subsequently as the source of purified [³H]vanillylamine.

Fig. 2.4.1.

Autoradiograph of [^3H]vanillylamine purified using 2D-TLC as described in section 2.6.2.3 . The vanillylamine (V) was located using the autoradiograph and subsequently extracted off the TLC plate into an appropriate buffer.
O = ORIGIN



2.4.2. Radiolabelling of Fruits and Cultured Cells of *C.frutescens* in vivo.

Several *in vivo* experiments in this investigation used radioactively labelled precursors of capsaicin. The labelled precursors were injected directly into the locus of attached fruits or added to cultured cells using a Hamilton microsyringe (100 μ l. capacity).

L-[U-¹⁴C]phenylalanine was obtained from Amersham International, Amersham, U.K. Aliquots, 1 μ Ci. in 20 μ l. stock solution, were used routinely. [³H]vanillylamine was custom-tritiated by Amersham International (section 2.4.2.). Aliquots (ca. 5 μ Ci. in 50 μ l. solution) were used routinely.

Harvesting took place at various time intervals after the addition of radiolabel depending on the nature of the experiment. For long incubation times (>2h.), the radiolabelled precursor was filter sterilized prior to use as described in section 2.2.2.4.

2.5. Extraction and Assay of Enzyme Activities.

2.5.1. Extraction of Enzyme Activities from Fruits and Cell Cultures of *C. frutescens*.

2.5.1.1. Extraction of phenylalanine ammonia-lyase activity.

An extraction technique was developed based on the methods of Hahlbrock and Wellmann, 1973 and Ozeki and Komamine, 1985.

Unless otherwise stated, all operations were carried out at 0–4°C. A known fresh weight of plant material (usually 2–3g) was ground in a mortar with 2% polyvinylpyrrolidone (PVP) in 3ml. of 0.2M. Tris-HCl, pH 8.8 containing 10mM β -mercaptoethanol. The PVP was added to remove phenolic impurities (Loomis and Battaile, 1966). Cell debris was removed by centrifugation at 2000xg. for 20 min. The supernatant was treated for 20 min. with 0.2g. Dowex 1x4 (Sigma), equilibrated with the same buffer as described above. After removal of the resin by centrifugation at 2000xg. for 20 min. the supernatant was used directly for the assay of phenylalanine ammonia-lyase.

2.5.1.2. The extraction of capsaicin synthase activity.

Measurements of the specific activity of capsaicin synthase were made on crude cell-free extracts obtained from either the fruits or cultured cells of *C. frutescens*. Unless otherwise indicated, all operations were carried out at 0–4°C.

Fruits of a selected age were harvested, weighed and then frozen in liquid nitrogen to facilitate extraction by grinding. Following freezing the fruits were ground in a mortar to a fine powder which was then stirred in extraction buffer (10ml g.f.wt.⁻¹ of Tris-HCl buffer, 0.1M, pH 8.0, containing 3mM ethylenediamine-tetra acetic acid (Na₂-EDTA), 10mM β-mercaptoethanol and 1mM phenylmethyl sulphonyl fluoride (PMSF)), with 2% polyvinylpyrrolidone (PVP) to remove phenolics from the extract, all chemicals were from Sigma. The extract was then homogenized for short bursts at full speed using a Sorvall Omni-mixer. After large tissue fragments had been removed by filtration through a single layer of Miracloth, the filtrate was centrifuged at 1000xg. for 20 min. to remove cell debris and PVP-bound phenolics. Since capsaicin negatively feeds back onto capsaicin synthase, it was necessary to remove the end product by Sephadex chromatography. After centrifugation, 2.5ml. of the supernatant was applied to a PD-10 Sephadex G-25M column (Pharmacia LKB, Milton Keynes, U.K.) which was equilibrated with the extraction buffer described above. The column was eluted with the same buffer and 0.1ml. fractions collected using an LKB fraction collector. All fractions containing protein (as determined by the method of Bradford, (1976). See section 2.5.2.4) were combined and used as the source of capsaicin synthase activity.

Cultured cells of *C. frutescens* were extracted in a slightly different way. Cells were not frozen in liquid nitrogen prior to extraction but ground immediately in buffer with a pestle and mortar, neither was the extract subjected to PD-10 Sephadex chromatography since no measurable levels of capsaicin were detected in cell cultures.

2.5.2. Assay of enzyme activities from fruits and cultured cells of *C. frutescens*.

2.5.2.1. Determination of the specific activity of phenylalanine ammonia-lyase.

The activity of phenylalanine ammonia-lyase was assayed using a procedure based on the methods of Zucker, (1965) and Tanaka *et al.*, (1974).

The extinction coefficient of trans-cinnamic acid dissolved in TRIS-HCl buffer at pH 8.8 is approximately 10,000 at 290nm, phenylalanine has almost no absorption at this wavelength. Consequently, the reaction catalysed by phenylalanine ammonia-lyase, which deaminates phenylalanine to form trans-cinnamic acid can be followed conveniently by measuring the increase in absorption at 290nm.

The reaction mixture consisted of 12mM L-phenylalanine; 150mM TRIS-HCl buffer, pH 8.8, 0.3ml. of enzyme extract (section 2.5.1.7) all in a total reaction volume of 4.3ml. The reaction was carried out at 40°C and was initiated by adding the substrate L-phenylalanine. Measurements of absorption at 290nm were made using a PYE Unicam SP500 Series2 spectrophotometer. Readings were taken at time zero and 2 h. after the addition of phenylalanine. The rate of reaction was found to remain constant for 4 h. and thereafter declined slowly. No increase in absorption was found when either phenylalanine or the enzyme was omitted from the reaction or when the enzyme was boiled prior to use. A reaction mixture without phenylalanine was used routinely as a control.

2.5.2.2. Determination of the specific activity of capsaicin synthase.

The formation of capsaicinoid from vanillylamine and 8-methylnonanoic acid by the enzyme(s) capsaicin synthase was assayed using two different assay systems described below. The first is a non-radioactive assay and the second utilizes [^3H]vanillylamine as one of the substrates.

[system 1]:

In this system all components added to the reaction mixture were non-radioactive. This assay relied on the fact that quantities of capsaicin measurable by HPLC would be produced during the incubation period.

The reaction tube contained the following components present at the final concentrations indicated (unless otherwise stated in the text); magnesium chloride (5mM), adenosine triphosphate (5mM), coenzyme A (1mM), vanillylamine hydrochloride (10mM), 8-methylnonanoic acid (10mM), Tris-HCl (0.1M) (containing 10mM β -mercaptoethanol) buffer, pH 7.4 and enzyme preparation (2-4mg. protein) in a total volume of 3ml. (all chemicals from Sigma). Substrates, cofactors and enzyme preparation were all brought to the assay temperature (30°C) and allowed to equilibrate before mixing. Boiled enzyme extract was used routinely as a control. The reaction was started by the addition of vanillylamine hydrochloride and was continued for 2h. The reaction was terminated by partitioning the reaction mixture with an equal volume of chloroform, this extracted the capsaicinoid and phenylpropanoid components into the organic phase. The extraction was completed as described in section 2.6.1.2 and the chloroform fraction subsequently analysed using HPLC (section 2.6.3.).

[system 2]:

In this system [^3H]vanillylamine (which had been purified as described in section 2.4.1.) was added to the reaction mixture and the incorporation of label from precursor into capsaicinoid was used as an indication of capsaicin synthase activity. The specific activity of the [^3H]vanillylamine

could not be determined due to the problems of self-radiolysis outlined in section 2.4.1. but the activity supplied (μCi) in each assay was known so the dpm into capsaicinoid could be expressed as a percentage of the total label supplied. This assay system was found to be more sensitive than system 1.

The reaction tube contained all the components described in system 1. except the cold vanillylamine hydrochloride which was replaced in system 2. by the purified [^3H]vanillylamine (usually *ca.* 5 μCi . in 100 μl . to each assay tube) added to start the reaction. The exact activity of [^3H]vanillylamine varied each time the purification was performed so the radioactivity was routinely measured using liquid scintillation counting (section 2.6.4.) prior to experimentation. All conditions were the same as described for system 1. and termination of the reaction along with subsequent extraction were performed as for the cold assay.

2.5.2.3. Estimation of the amount of soluble protein present in the enzymatic extract.

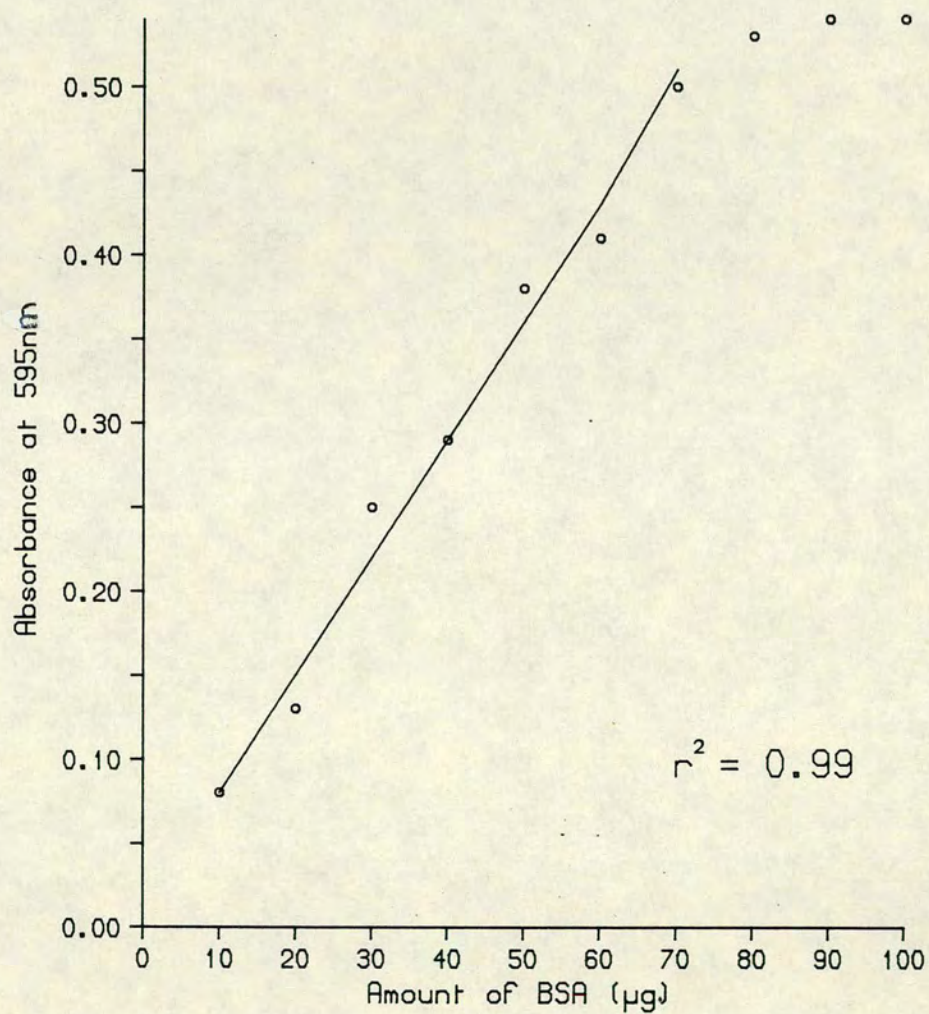
The protein content of the enzyme extract was determined using the method of Bradford, (1976). The reagent was prepared by dissolving 100mg. of Coomassie Brilliant Blue G-250 (Sigma) in 50ml. 95% ethanol and adding 100ml. of orthophosphoric acid. The resulting solution was diluted to a final volume of 1 litre, filtered through Whatman 1 filter paper and stored in a dark glass bottle. The reagent was stored for up to 2 weeks and then discarded.

From the enzyme extract a 0.5ml. sample was taken and mixed with 0.02ml. of 100% trichloroacetic acid (TCA). This was allowed to stand at 0–4°C for 24h. to allow the TCA to precipitate the soluble protein (Lowry *et al.*, 1951). The sample was then centrifuged for 5 min. at 2000 $\times g$, the supernatant discarded and the pellet resuspended in 1.0ml. of 0.1N NaOH. This was left to stand at room temperature for 30 min. The sample was diluted as required and 0.1ml was thoroughly mixed with 3.0ml. of dye reagent (as described above). After 5 min. the absorbance was measured at 595nm using a Pye Unicam SP500 Series 2 Spectrophotometer. Protein concentration was estimated using a bovine serum albumin (BSA) (Sigma) calibration curve (see Fig. 2.5.2.).

Fig. 2.5.2.

Relationship between absorbance at 595nm and amount of BSA, where $r^2 =$ correlation coefficient of the fitted line.

Fig. 2.5.2.



2.6. Analytical Techniques.

2.6.1. Extraction of Capsaicinoids and phenylpropanoids.

2.6.1.1. Extraction of capsaicinoids and phenylpropanoids from fruits and cultured cells of *C. frutescens*.

Capsaicinoids were extracted from fruit tissue and from cell cultures according to the method described by Hall *et al.*, (1987). Extracted along with the capsaicinoids were all the other phenylpropanoids of the capsaicin biosynthetic pathway. The efficiency of this extraction procedure was checked for each of the intermediates (see Table 2.6.1.1).

Table 2.6.2.1

The efficiency of the methanol extraction procedure for the extraction of phenylpropanoids and capsaicinoids from cells of *C. frutescens*.

compound	%recovery in the methanol fraction				
	rep(1)	rep(2)	rep(3)	mean	se.
cinnamate	69	73	76	72.7	± 2.03
coumarate	65	65	65	65.0	± 0.00
caffeate	66	68	69	67.7	± 0.88
ferulate	66	72	70	69.3	± 1.76
vanillin	73	72	66	70.3	± 2.19
vanillylamine	77	82	76	78.3	± 1.86
dihydrocapsaicin	76	69	68	71.0	± 2.52
capsaicin	65	69	66	66.7	± 1.20

where rep = replicate

The plant material was harvested, weighed and then thoroughly homogenized in a large volume of methanol (*ca.* 10ml. g.f.wt.⁻¹) using a pestle and mortar. The homogenate was stirred under nitrogen in darkness for 4h. after which time the methanol extract was removed by filtration through a glass-fibre filter (Whatman, U.K.). The procedure was repeated with a similar volume of fresh methanol and left to stir overnight. Where plant material had been subjected to radio-labelling, the extraction procedure was carried out in the presence of added non-radioactive carriers (0.1mg. of each of the phenylpropanoids) to optimize the extraction efficiency of the labelled phenylpropanoids. The combined extract was dried *in vacuo* at 35°C and the residue taken up in 1ml.

of methanol (HPLC-grade) for 1h. on ice.

2.6.1.2. Extraction of capsaicinoids and phenylpropanoids from aqueous fractions.

Capsaicinoids and phenylpropanoids were extracted from cell culture medium after removal of the cells by filtration. The compounds were extracted three times into chloroform (each volume equal to the aqueous volume) using a separating funnel. The extract was subsequently dried using *ca.* 10g.50ml⁻¹ of anhydrous sodium sulphate (BDH) and after filtration was dried in vacuo at 30°C and the residue taken up in 1ml, methanol (HPLC-grade) for 1 hour on ice. The extraction efficiency of this procedure was found to be over 96% efficient for all intermediates except vanillylamine and dihydrocapsaicin. The extraction efficiency of these two compounds was lower (see Table 2.6.1.2).

Table 2.6.1.2

The efficiency of the chloroform extraction procedure for the extraction of phenylpropanoids and capsaicinoids from liquid medium and other aqueous fractions.

compound	% recovery in the chloroform fraction				
	rep(1)	rep(2)	rep(3)	mean	se.
cinnamate	104	99	100	101.0	± 1.53
coumarate	98	97	98	97.7	± 0.33
caffeate	96	100	95	97.0	± 1.53
ferulate	98	99	99	98.7	± 0.33
vanillin	94	98	97	96.3	± 1.20
vanillylamine	5	15	6	8.7	± 3.18
dihydrocapsaicin	62	69	65	65.3	± 2.03
capsaicin	106	99	98	101.0	± 2.52

where rep = replicate

Capsaicinoids and phenylpropanoids were extracted from the reaction mixture following termination of the reaction catalysed by capsaicin synthase (see section 2.5.2.2). The same extraction procedure was employed for this aqueous system as for the culture medium extraction described above. This method conveniently separated the substrate vanillylamine, from the product capsaicinoid.

2.6.2. Analysis of Capsaicinoids by Thin Layer Chromatography.

Thin layer chromatography was used for the qualitative and semi-quantitative analysis of extracts.

2.6.2.1. One-dimensional Thin Layer Chromatography.

One dimensional TLC was used regularly for preliminary analysis of extracts from radiolabelling studies. Since the [^3H]vanillylamine remained at the origin in the solvent system used (benzene: glacial acetic acid, 9:2) and all other intermediates of the pathway moved away from the origin, the detection of other labelled peaks could be made quickly and with a high degree of resolution using a Radioactivity intelligent thin-layer analyser (RITA, section 2.6.2.2). However, intermediates of the capsaicin biosynthetic pathway could not be completely separated and identified using one-dimensional TLC since several of the molecules co-elute. If radiolabel was found in any region other than the origin, then further rigorous analysis using 2D-TLC (section 2.6.2.3), autoradiography (section 2.6.2.4) and HPLC (section 2.6.3.) was performed. One-dimensional TLC thus served as a quick and effective method of identifying those assays which had produced a positive result from those which had not.

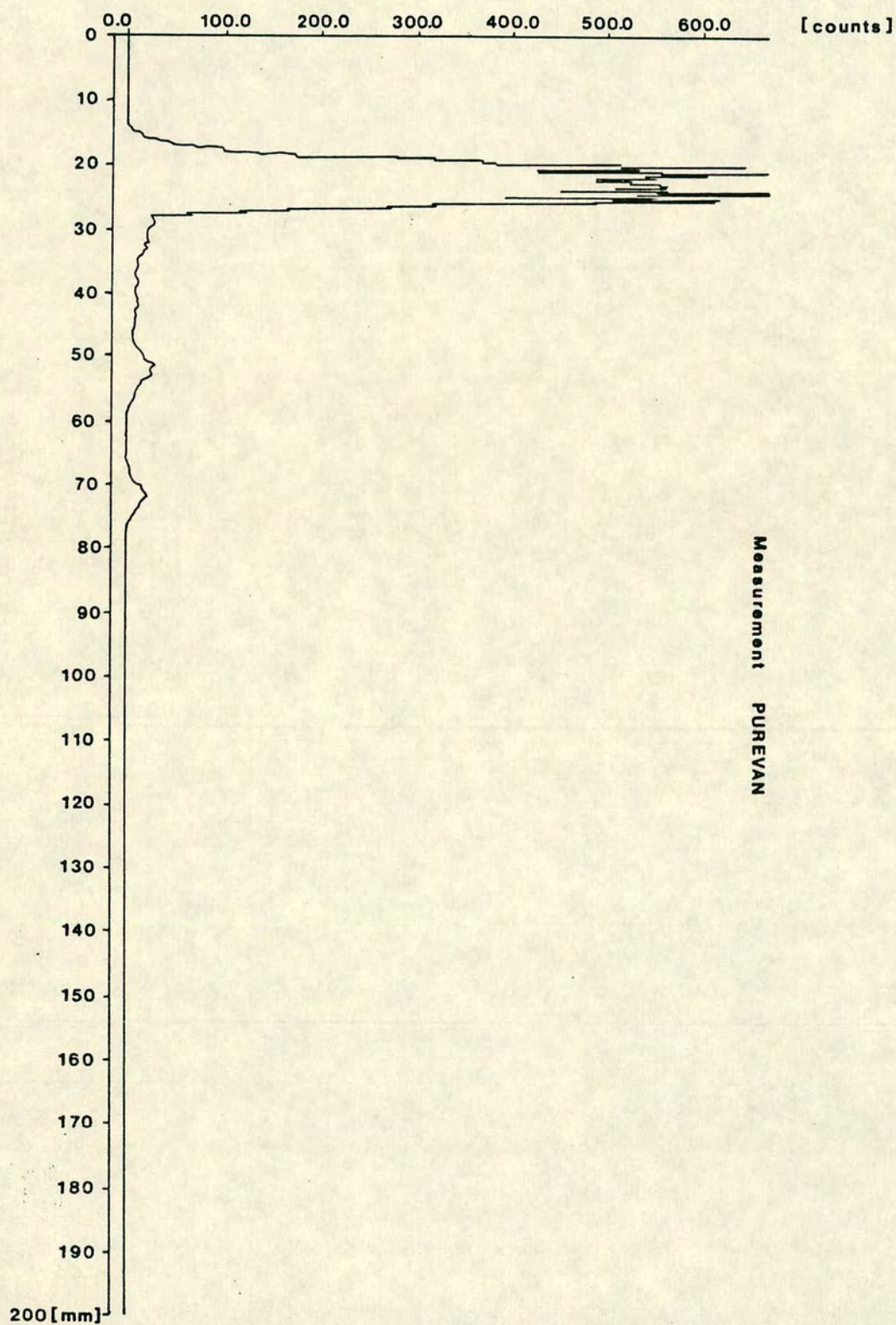
Plastic backed Kieselgel-60 plates (Merck, W.Germany) were loaded using a Hamilton glass syringe (capacity 100 μl). Sealed TLC tanks were equilibrated with the solvent system 1-2h. prior to use. The loaded plates were run for 1-1.5h. in the solvent, removed and allowed to dry thoroughly before further analysis using the RITA.

2.6.2.2. Radioactivity Intelligent Thin-layer Analysis (RITA.)

In some cases one-dimensional TLC was used to separate radioactively labelled molecules (section 2.6.2.1). The radioactive spots on the TLC plates were detected using a radioactivity intelligent thin-layer analyzer (RITA).

The RITA functions in the following way; The radiation particles released from the sample cause localized ionization of a gaseous mixture of methane (10%) and argon (90%) which pass through the detector head over the surface of the TLC plate. A strong electrical field is generated by the presence of a high voltage counting wire which effectively amplifies the signal into an

ISOMESS IM-3000 Radio-TLC-Analyzer, Time: 600 s.



electrical charge by secondary ionization. The generated electrical signal runs in both directions from the point of origin along the counting wire and a comparison is made by the computer of the times of arrival of the signal at both ends of the detector head . This gives positional information about the localization of the radioactive spots. A typical RITA trace of [^3H]vanillylamine run one dimensionally can be seen in Fig. 2.6.2.2.

Fig. 2.6.2.2

RITA trace of purified [^3H]vanillylamine (V) which had been subjected to 1D-TLC as described previously.

2.6.2.3 Two-dimensional TLC.

The following 2D-TLC system was used routinely to separate all the phenolic intermediates of the capsaicin biosynthetic pathway with a high degree of resolution, Fig. 2.6.2.3. Plastic backed Kieselgel-60 TLC plates (Merck, W.Germany) were used with the solvents benzene: glacial acetic acid (9:2, 1st dimension) and butanol: iso propanol: ammonia: water (2:6:1:1, 2nd dimension). Sealed TLC tanks were equilibrated with the solvent system 1-2h. prior to use.

Loaded TLC plates were eluted for 1-1.5h. in the first solvent system, removed and thoroughly dried before elution in the second solvent system for 5h. Plates were allowed to stand for 24h. in a fume cupboard, when dry, they were sprayed with a solution containing 0.1g·FeCl₃·6H₂O and 0.05g·K₃Fe(CN)₆ in 10ml.of distilled water (freshly made each time) to localize the phenolics.

The phenylpropanoids and capsaicinoids were identified by comparing the R_f. values of standards (Sigma) run in the same solvent systems.

2.6.2.4. Autoradiography.

Autoradiography was carried out on TLC plates which had been subjected to the 2D-TLC protocol outlined above (2.6.2.3) when the extract to be analysed was radioactive. Localization of the intermediates with a reagent was performed after autoradiography in these cases since quenching due to the dye reduced the efficiency of the autoradiography.

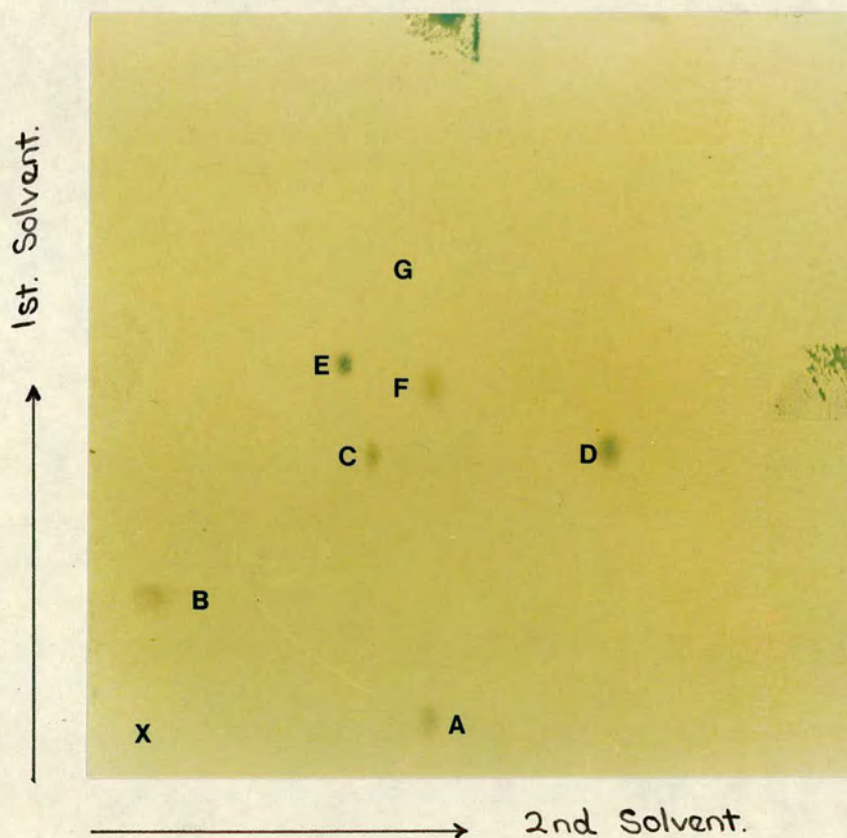
Autoradiography was carried out at -40°C using Hyperfilm-MP (Amersham, U.K.) which had been pre-flashed twice prior to use. The exposure time was usually 2 weeks. The cassette-enclosed film was allowed to reach room temperature before development using a Gevomatic 60 automatic developer (Agfa-Gevaert, W.Germany).

Fig. 2.6.2.3

Two-dimensional TLC of the phenylpropanoids and capsaicin. Cinnamate cannot be seen on this particular plate since the plate had been exposed to light for too long and the background had faded to the colour of the cinnamate spot.

where:

A	= vanillylamine
B	= caffeic acid
C	= coumaric acid
D	= capsaicin
E	= ferulic acid
F	= vanillin
G	= cinnamate
X	= ORIGIN



2.6.3. High Performance Liquid Chromatography.

High performance liquid chromatography (HPLC) was used to perform qualitative and quantitative analysis of extracts. It was also used to fractionate radioactive extracts for subsequent liquid scintillation counting analysis, section 2.6.4.

2.6.3.1. The HPLC System.

High performance liquid chromatography was performed according to the method described by Hall *et al.*, (1987).

A Hewlett-Packard HP1090 liquid chromatograph fitted with autosampler and binary solvent delivery system was used in conjunction with an HP1040 diode array detector (Hewlett-Packard, Reading, U.K.) fitted with a 4.5µl. flow cell. Separation was achieved using a column (200x5mm, 5µm. Spherisorb C8) (Phase Separations, Clwyd, U.K.) at 40°C with a varying mixture of HPLC grade methanol and 5% acetic acid in water (BDH, Poole, U.K.) as eluting solvent.

2.6.3.2. Preparation of the mobile phase.

The solvents used were filtered under vacuum to remove particles which could block and damage the HPLC pumps and tubing. Methanol was filtered through 0.45µm-pore Nylon-66 membrane filters (Anachem, Luton, U.K.) and the aqueous mixture of 5% acetic acid was filtered through 0.45µm-pore cellulose acetate membrane filters (Schleicher and Schull, Surrey, U.K.). Once the solvents had been filtered, they were then degassed by gently bubbling helium through them from a sparger for 10min., this helped to prevent the formation of air bubbles within the HPLC equipment.

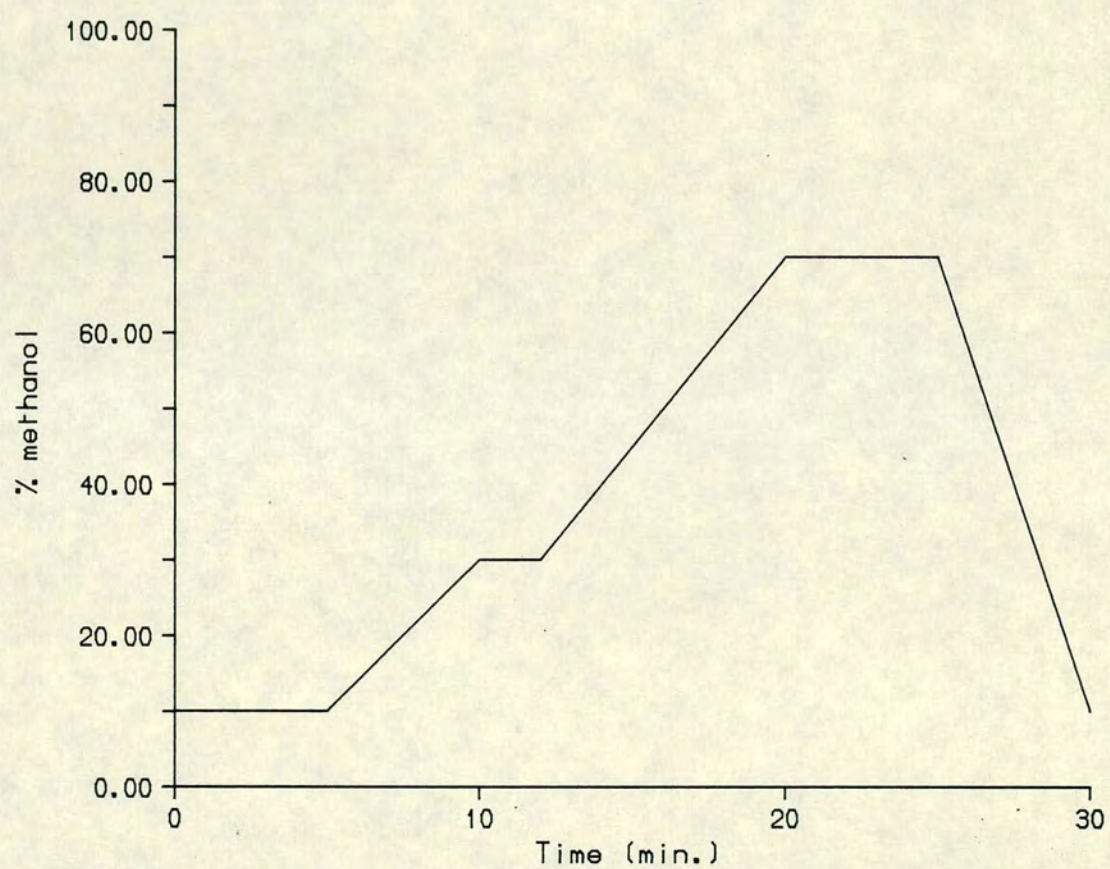
2.6.3.3. Preparation of samples for HPLC analysis.

Samples were stored in 1ml. of HPLC-grade methanol at 0-4°C and were analysed as soon as was reasonably possible following extraction (usually within 24h.). The samples were filtered prior to HPLC analysis to remove particulate contaminants. All samples were filtered using a microfilter (Bioanalytical Systems) fitted with a 0.45µm. Nylon-66 membrane filter (Rainin) by centrifugation at 1000xg. for 5min. The filtered sample was then transferred to a 2ml. autosampler vial (Chromacol, London.) and sealed with a crimp top.

Fig. 2.6.3.4 a.

The solvent gradient profile used for the elution of phenylpropanoids and capsaicinoids.

Fig. 2.6.3.(4)a.



2.6.3.4. HPLC analysis of samples.

Samples and solvents were prepared as described in sections 2.6.3.3 and 2.6.3.2 respectively. The HPLC system was as described in section 2.6.3.1. The column was eluted with the mobile phase mixture at a rate of 1 ml min^{-1} according to the gradient system shown in Fig. 2.6.3.4a. (Hall *et al.*, 1987). Detection of eluted phenylpropanoids and capsaicinoids was achieved at 280nm. A chromatogram of a mixture of authentic phenylpropanoid and capsaicinoid standards is shown in Fig. 2.6.3.4b. Output from the diode array detector was stored on disc, this facilitated UV spectral analysis of individual compounds.

Calibration curves of peak area plotted against amount of compound were prepared for each of the intermediates of the capsaicin biosynthetic pathway and for capsaicin (Fig. 2.6.3.4c.). These curves were then used in the subsequent calculation of intermediate and capsaicinoid content of tissues.

2.6.3.5. Collection of compounds eluted from the HPLC column.

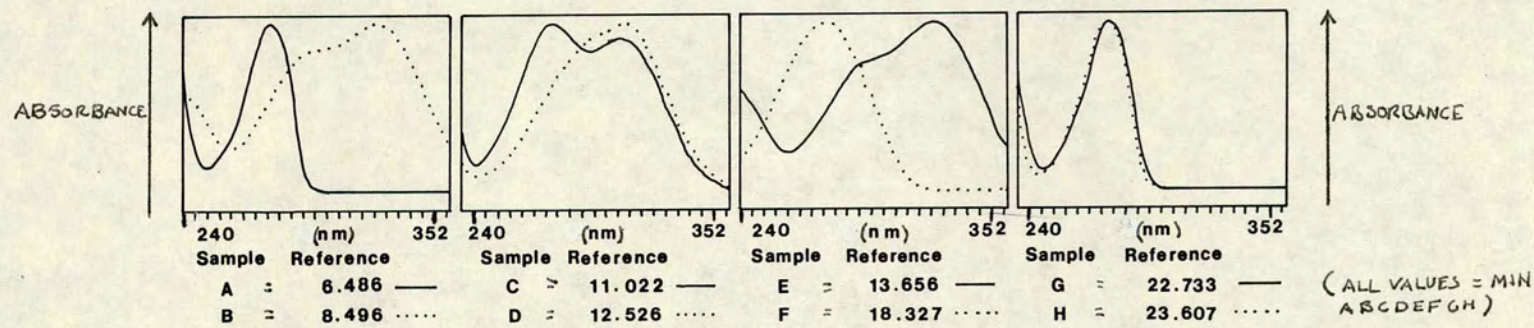
In studies involving the use of radioactively labelled precursors, it was necessary in some cases to locate the radioactivity using HPLC separation and fraction collection. Once the retention times had been determined for each of the compounds, a programmable LKB 2211 superrac fraction collector (LKB, Croydon) was used to collect the fractions as they were eluted from the column. The fraction collector was connected to the outlet from the Hewlett-Packard flow cell and a delay was included in the programme to account for the time between UV detection of a compound and its subsequent collection. Fractions were then counted using liquid scintillation counting as described in section 2.6.4.

Fig. 2.6.3.4b.

A diagrammatic chromatograph of a standard solution of capsaicin and phenylpropanoid compounds (bottom). The spectra (240–352nm) of the eluted compounds are drawn above and the compounds are identified on the diagram.

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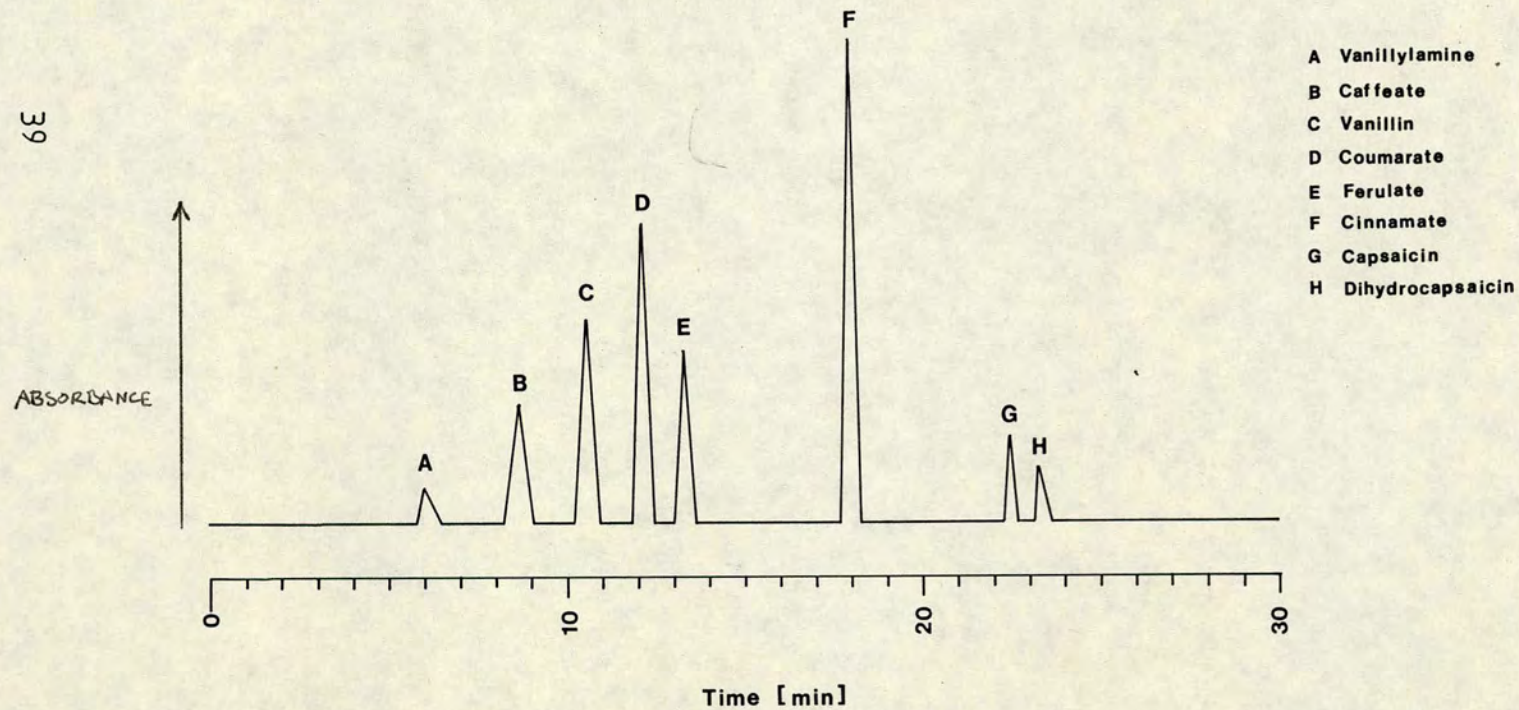
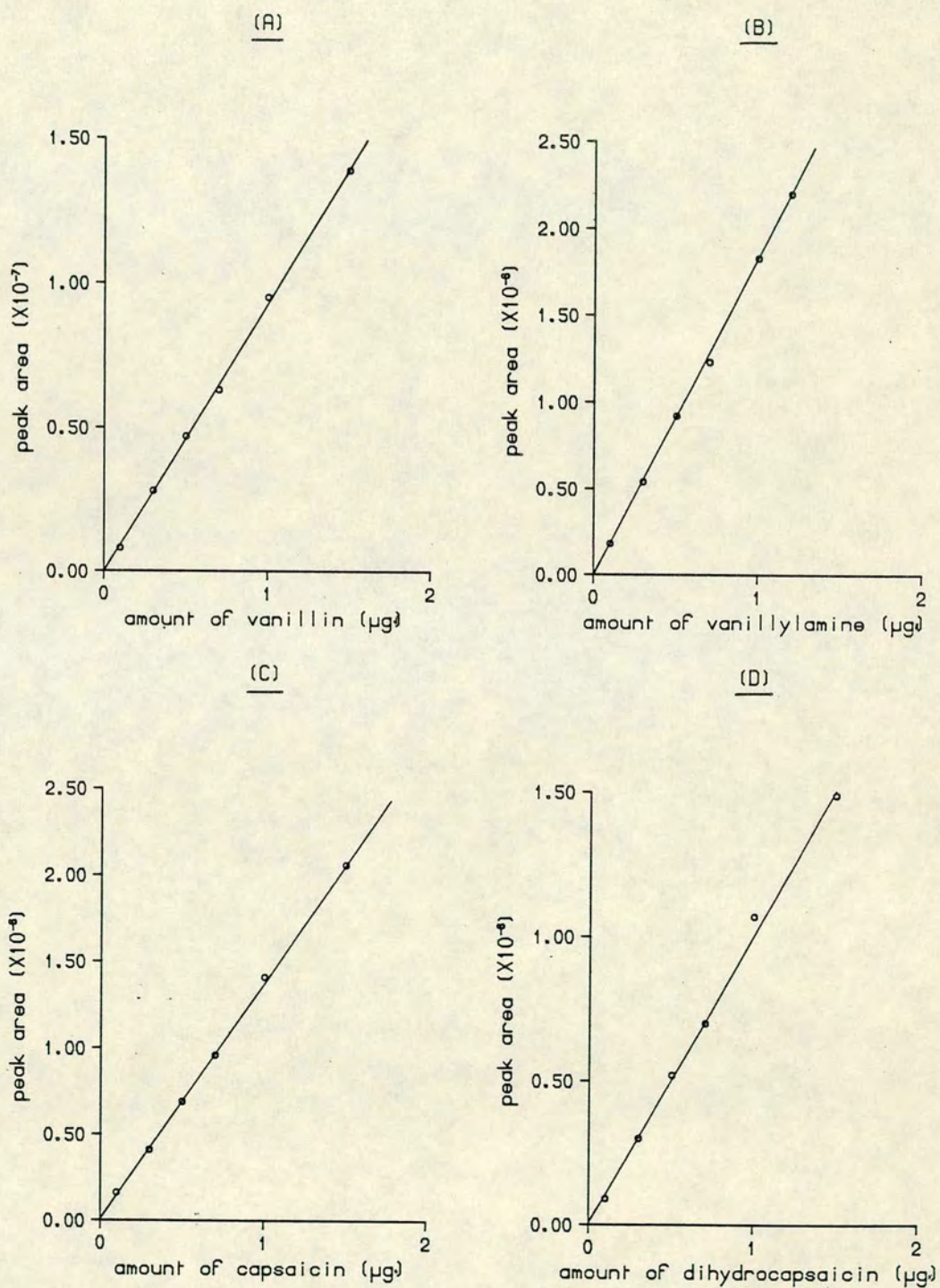


Fig. 2.6.3.4c.

HPLC calibration curves for vanillin (A), vanillylamine (B), capsaicin (C) and dihydrocapsaicin (D).



2.6.4. Liquid Scintillation Counting.

The presence of radioactivity in [³H]vanillylamine, [¹⁴C]phenylalanine or subsequently in enzymatic products was determined by liquid scintillation counting.

2.6.4.1. The scintillation cocktails.

For organic soluble samples the scintillation cocktail was prepared as follows: The primary solute butyl-PBD (2.4g) was dissolved in a mixture of the solvent toluene (200ml) and the emulsifier Triton X100 (100ml) (all chemicals from BDH, Poole, Dorset). The mixture was freshly prepared prior to use and was stored in a dark glass bottle for up to two days at 0–4°C.

For aqueous samples the scintillation cocktail was prepared as follows: The primary solute PPO (4.0g) and the secondary solute Me₂POPOP (0.2g) were dissolved in a litre of the solvent 1,4-dioxan. The mixture was made up immediately prior to use.

2.6.4.2. Liquid scintillation counting.

The radioactive sample was placed in a polythene vial together with 4ml of the appropriate scintillation cocktail. The sample was counted in an Intertechnique SC3000 scintillation counter for 10min. at an error of 0.5%. The results were obtained as counts per minute (cpm) which were then converted to disintegrations per minute (dpm) using the external standards ratio method. The quench correction curves seen in Fig. 2.6.4.2a. and 2.6.4.2b. were obtained by counting a known activity of the relevant isotope, and then sequentially quenching the sample with aliquots of acetone. Different X-values were obtained as the amount of quenching due to the acetone increased. In this way it was possible to plot percentage counting efficiency against the X-value as a quench correction curve which was used to convert cpm. to dpm. for all samples counted subsequently.

Fig. 2.6.4.2 a.

Quench correction curve for ^3H obtained by counting samples of ^3H vanillylamine quenched to varying degrees with acetone.

Fig. 2.6.4. (2) a.

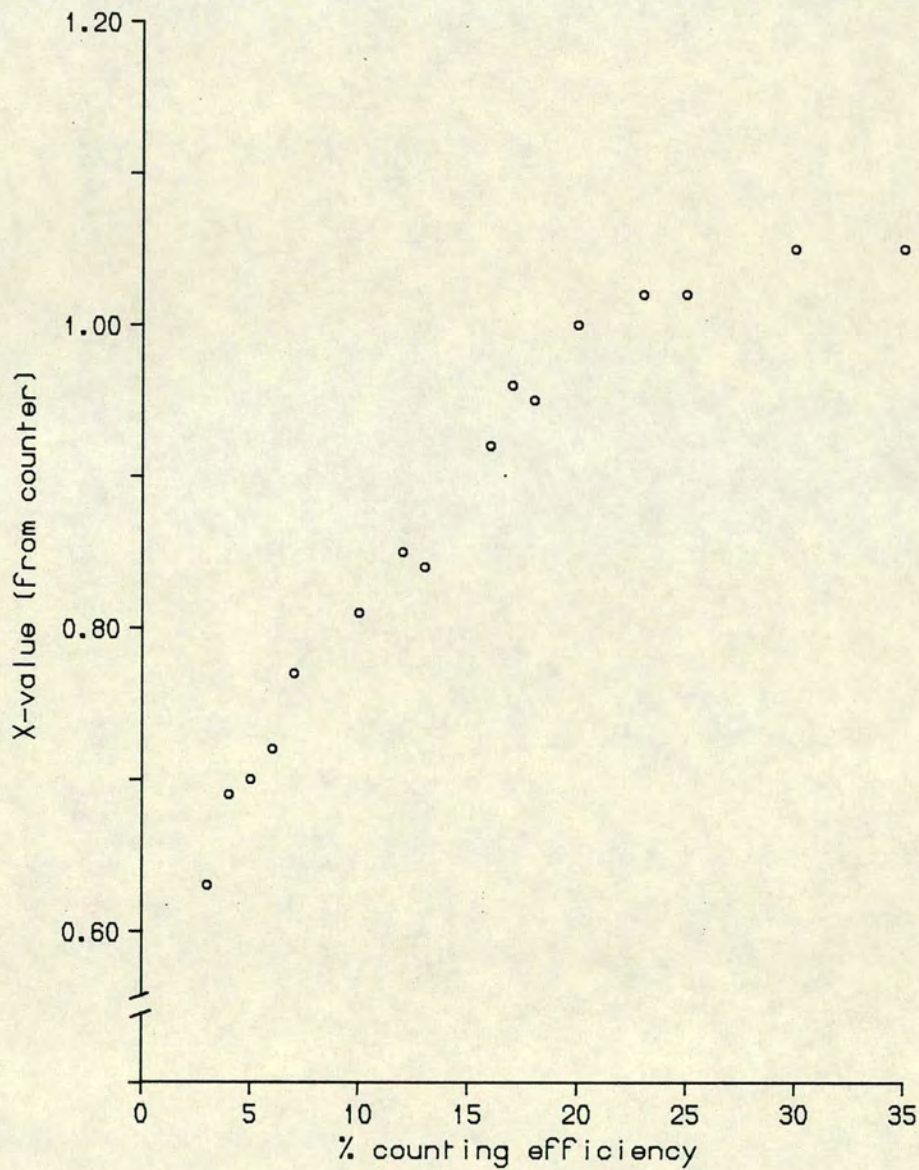
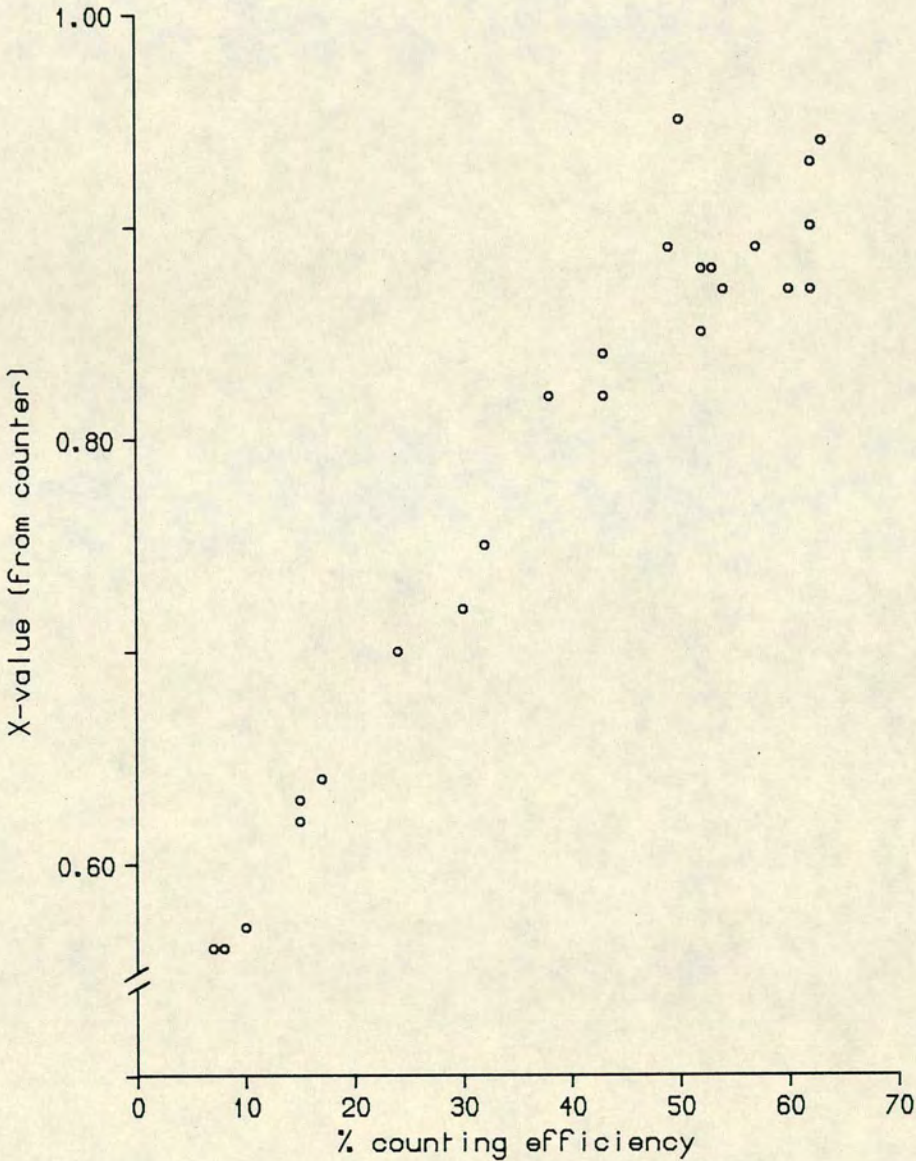


Fig. 2.6.4.2b.

Quench correction curve for ^{14}C obtained by counting [^{14}C]-phenylalanine quenched to various degrees with acetone.

Fig. 2.6.4.(2)b.



2.7. Mathematical Analysis of Results.

2.7.1. Calculation of the Specific Activity of PAL.

A molar solution of cinnamate has an optical density of 10,000 at 290nm. That is to say, the extinction coefficient of cinnamate at 290nm is 10,000.

During the incubation of the assay mixture there is a change in the optical density due to the formation of cinnamate:

1)..... $\Delta OD = A$

In the assay tube an amount of cinnamate has been produced:

2)..... $A/10,000 \text{ moles l}^{-1}$

So in the reaction vessel of total volume Vml. this amount of cinnamate has been formed:

3)..... $A/10,000 \times V/1,000 \text{ moles}$

The rate of cinnamate synthesis can be represented as follows:

4)..... $A \times V/(10,000,000 \times t) \text{ moles s}^{-1}$

All assays used 0.3ml. of enzyme extract which contained P μ g. of protein, the specific activity of phenylalanine ammonia-lyase can now be calculated by dividing the rate of cinnamate formation by the amount of protein:

5)..... $A \times V/10,000,000 \times t \times P \text{ moles s}^{-1} \mu\text{g. P}^{-1}$

Since the specific activity of an enzyme is usually expressed in $\mu\text{kat.kg}^{-1}$, the final calculation is as follows:

6)..... $(A \times V/10,000,000 \times t \times P) \times 10^6 \times 10^9 \mu\text{Kat.kg}^{-1}$

2.7.2. Determination of the standard error of a mean.

In the majority of experiments three replicates per treatment were used and the mean of these three values calculated. The standard error of the mean was calculated to determine the amount of variation within a treatment. The standard error was calculated according to the method of Parker (1979).

2.7.3. Comparison of the means of two samples

Observed differences between means were determined using "Student's t-test" as it is suitable for the small samples ($n \leq 30$) used in this investigation.

The first sample contained n_1 observations and had a mean of \bar{x}_1 and a variance s_1^2 . In the second sample the corresponding values were n_2 , \bar{x}_2 and s_2^2 . The null hypothesis was tested that the true means μ_1 and μ_2 were equal. The common sample variance was calculated and this value was used to calculate t . The table of t distribution was then referred to (Bailey, 1981) at $(n_1 + n_2 - 2)$ degrees of freedom. If t was bigger than the tabulated figure corresponding to P , the hypothesis of equal means was rejected at the 100P % level of significance, otherwise it was accepted at 100P %.

Chapter 3

Experimental Results

The regulation of capsaicin biosynthesis has been studied in this thesis. Particular attention has been given to the role of selected enzymes in the control of the pathway focussing on the terminal reaction sequence.

At the start of this investigation available evidence suggested that the synthesis and accumulation of capsaicin in the fruit and in cultured cells of *C.frutescens* was probably regulated by reactions at the end of the biosynthetic pathway and not by enzymes at the beginning. Before proceeding to examine in detail the reactions at the end of the pathway where control was probably sited, it was necessary to confirm this earlier assertion that PAL and C,4-H were not involved in the regulation of capsaicin synthesis.

In Part 3.1. experiments concentrate on PAL and C,4-H, the first two enzymes in the capsaicin biosynthetic pathway. Techniques required for the study of other enzymes in the pathway were also developed. In addition, a series of experiments utilizing the selective inhibitor of PAL, AOPP (amino oxyphenyl propionate), was carried out to provide further information on the regulation of capsaicin biosynthesis.

Experiments in Part 3.2. quantify the uptake and conversion of [³H]vanillylamine into capsaicinoids *in vivo* in order to: a) confirm the presence of the terminal enzyme reaction in fruits and cultured cells of *C.frutescens* and b) test the suitability of [³H] vanillylamine for use in an *in vitro* assay.

In Part 3.3. the development of an assay for capsaicin synthase in extracts from fruits of *C.frutescens* is described. Fruits were used as they are easy to grow and contain significant quantities of the enzyme activity at a known stage of development. Partial characterization of the reactions catalysed by capsaicin synthase was achieved in Part 3.4.

In Part 3.5. a correlation was made of capsaicin synthase activity with the onset of capsaicin accumulation in fruits of *C.frutescens*.

The effects of selected culture regimes and stress treatments on the activity of capsaicin synthase were investigated using the *in vitro* assay system in Part 3.6.

Part 3.1. Determination of the Probable Rate-limiting Step for the Biosynthesis of Capsaicin in *C.frutescens*.

The experiments in this section concentrate on PAL and C,4-H, the first two enzymes in the capsaicin biosynthetic pathway. As both of these enzymes have been recognized as potential sites for regulation in other systems (Hahlbrock and Wellman, 1970 1973) they have been selected for study in cultures of *C.frutescens*. Two of the experiments in this section were designed to attempt to correlate levels of activity of the two enzymes with the onset of capsaicin synthesis in fruits and cultured cells. The third experiment utilizes the selective inhibitor of PAL, AOPP to provide further information on the regulation of capsaicin biosynthesis. These experiments were all carried out to confirm the assertion that PAL and C,4-H were not involved in the regulation of capsaicin synthesis.

3.1.1. Comparison of the specific activities of PAL and C,4-H in suspended and immobilized cells of *C.frutescens* throughout a culture cycle in relation to the incorporation of label from [¹⁴C]phenylalanine into capsaicin.

It has been shown that immobilized cells of *C.frutescens* have a greater capacity for incorporating [¹⁴C]phenylalanine into capsaicin than suspended cells (Lindsey, 1986). The present experiment was designed to measure the incorporation of radiolabel from [¹⁴C]phenylalanine into capsaicin and to see if the increase in flux through the pathway was reflected in a difference between the specific activities of phenylalanine ammonia-lyase, cinnamate 4-hydroxylase, or both of these enzymes in suspended and immobilized cells. Cultured cells of *C.frutescens* were used in this experiment for a number of reasons; a) cells can be easily manipulated in the cultured system, b) PAL and C,4-H assays had been optimized for extracts obtained from cultured cells, and c) differential levels of capsaicin accumulation had been observed in cells cultured under different conditions.

Cells of *C.frutescens* immobilized (1 block) or suspended (ca. 2.0g) were subcultured from one flask into standard media in 250ml. Erlenmeyer flasks at time = 0 and returned to an orbital shaker (section 2.2.3.1). One hour prior to harvest, 3 out of 6 replicates were supplied with [^{14}C] phenylalanine (1 μCi . per flask). At selected time intervals after subculture the cells were harvested. The radioactively labelled cells were extracted in methanol as described in section 2.6.1.1 and analysed using 2D-TLC and liquid scintillation counting (sections 2.6.2.3 and 2.6.4.). The radioactive media were extracted in chloroform (section 2.6.1.2) and analysed as for the methanolic extracts. The unlabelled cultures were divided, half of the cells were extracted and assayed for PAL activity (sections 2.5.1.1 and 2.5.2.1), whilst the remainder were extracted and assayed for C,4-H activity according to the method of Lamb and Rubery, (1975). The specific activities of PAL and C,4-H were calculated (section 2.7.1.) following determination of the soluble protein content of the enzymatic extracts (section 2.5.2.3).

Fig. 3.1.1a.

The effect of immobilization on the specific activities of phenylalanine ammonia-lyase (PAL) and cinnamate 4-hydroxylase (C,4-H) in cells of *C.frutescens* throughout a culture cycle. Each point represents the mean of three replicates \pm standard error. Where S.A. = specific activity.

Fig. 3.1.1b.

Incorporation of [^{14}C]phenylalanine into capsaicin in immobilized cells (squares) and freely suspended cells (circles) over a 32d. culture cycle. Each point represents the mean of three replicates \pm the standard error.

Fig. 3.1.1a.

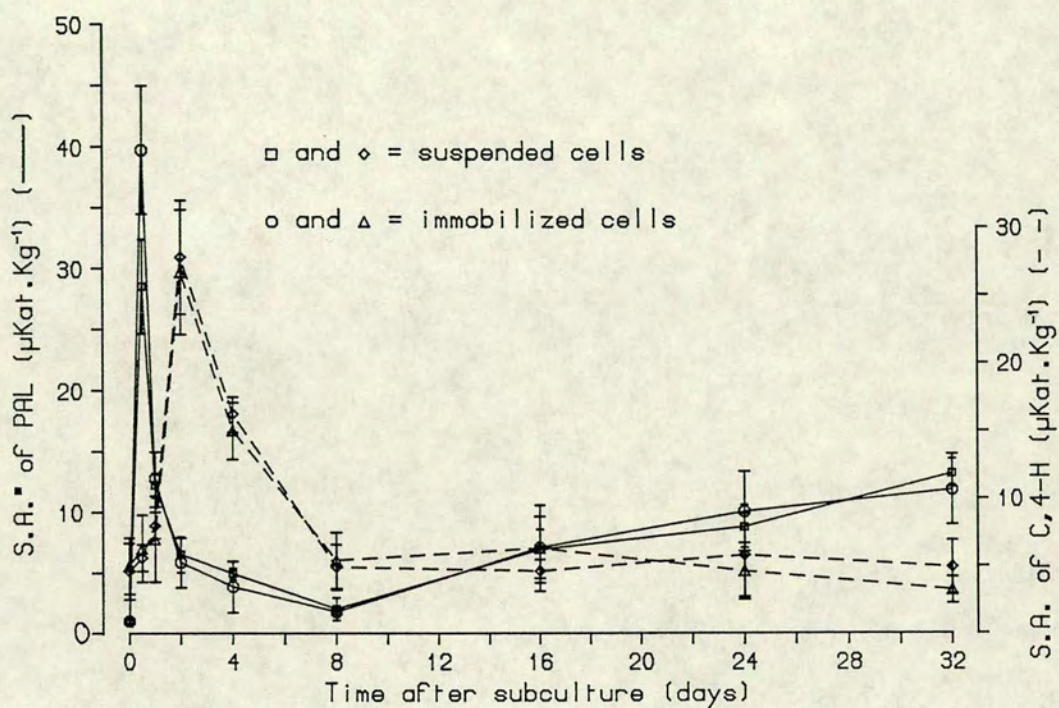
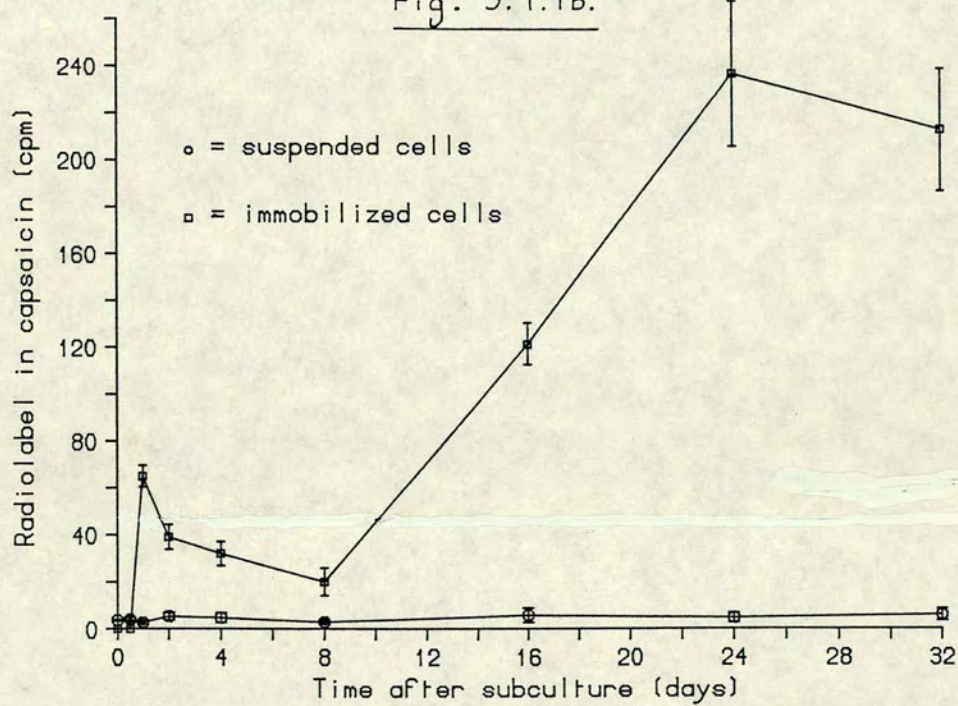


Fig. 3.1.1b.



It can be seen from Fig. 3.1.1a. that the specific activities of PAL and C,4-H were not significantly different between suspended and immobilized cells of *C.frutescens* throughout a culture cycle. Approximately 15h. after subculture there was a large increase in the specific activity of PAL which was closely followed by a significant increase in the specific activity of C 4-H in both suspended and immobilized cells. The specific activities of both enzymes had returned to their initial levels by day 4 and remained at those levels for the duration of the time course.

Fig. 3.1.1b. shows the incorporation of radiolabel from [^{14}C]phenylalanine into capsaicin throughout a culture cycle in immobilized and suspended cells. It can be seen that in suspended cells there was no detectable incorporation of radiolabel from [^{14}C] phenylalanine into capsaicin, whereas in immobilized cells there was a marked increase in the extent of incorporation into capsaicin between 8d. and 12d. after subculture. The increased incorporation into capsaicin in immobilized cells levelled off after 24 d.

These data show that the increased amount of incorporation of [^{14}C]phenylalanine into capsaicin in immobilized cells of *C.frutescens* is not paralleled by an increase in the specific activities of PAL or C,4-H in immobilized cells or suspended cells. The data presented in section 3.1.1. suggest that increased incorporation of [^{14}C]phenylalanine into capsaicin in immobilized cells is not related to the specific activities of PAL or C,4-H in cultured cells of *C.frutescens*. Quantities of capsaicin measurable by HPLC were not detected in the media or the cells in section 3.1.1. and the incorporation represents a very small amount of the net synthesis of capsaicin. Use of intact fruits of *C.frutescens* should overcome this problem and establish whether there is any relationship between PAL activity and capsaicin synthesis and accumulation. Fruits were used since; a) they produce large quantities of capsaicin and b) there is a very clear "switch-on" of capsaicin production *ca.* 20d. after fruit initiation.



3.1.2. Measurement of the specific activity of PAL throughout fruit development in relation to the onset of capsaicin production.

The aim of this experiment was to measure the specific activity of PAL throughout a time course of fruit development and to attempt to correlate changes in the specific activity of PAL with the onset of capsaicin accumulation.

Fruits (2 at each selected age (Part 2.1.)) were harvested and weighed. One was extracted for PAL activity (section 2.5.1.7) and the enzyme assayed as described in section 2.5.2.7. The specific activity of PAL was calculated (section 2.7.1. following determination of the soluble protein content of the enzymatic extract (section 2.5.2.3). The second fruit was dissected into placenta, pericarp and seed fractions and each was separately extracted in methanol for capsaicin as described in section 2.6.1.7. The capsaicin content was determined using HPLC (section 2.6.3.). Three replicates at each fruit age were used in this experiment.

Fig. 3.1.2a.

The accumulation of capsaicin in whole fruits (squares), in placentae (circles) and in pericarp (triangles) throughout a time course of fruit development. Each point represents the mean of three replicates \pm standard error. No detectable levels of capsaicin were found in the seeds of *C.frutescens*, these results are not included here.

Fig. 3.1.2b.

The specific activity of PAL throughout a time course of fruit development in relation to the total capsaicin accumulated by fruits of *C.frutescens*. Each point represents the mean of three replicates \pm standard error.

Fig. 3.1.2a.

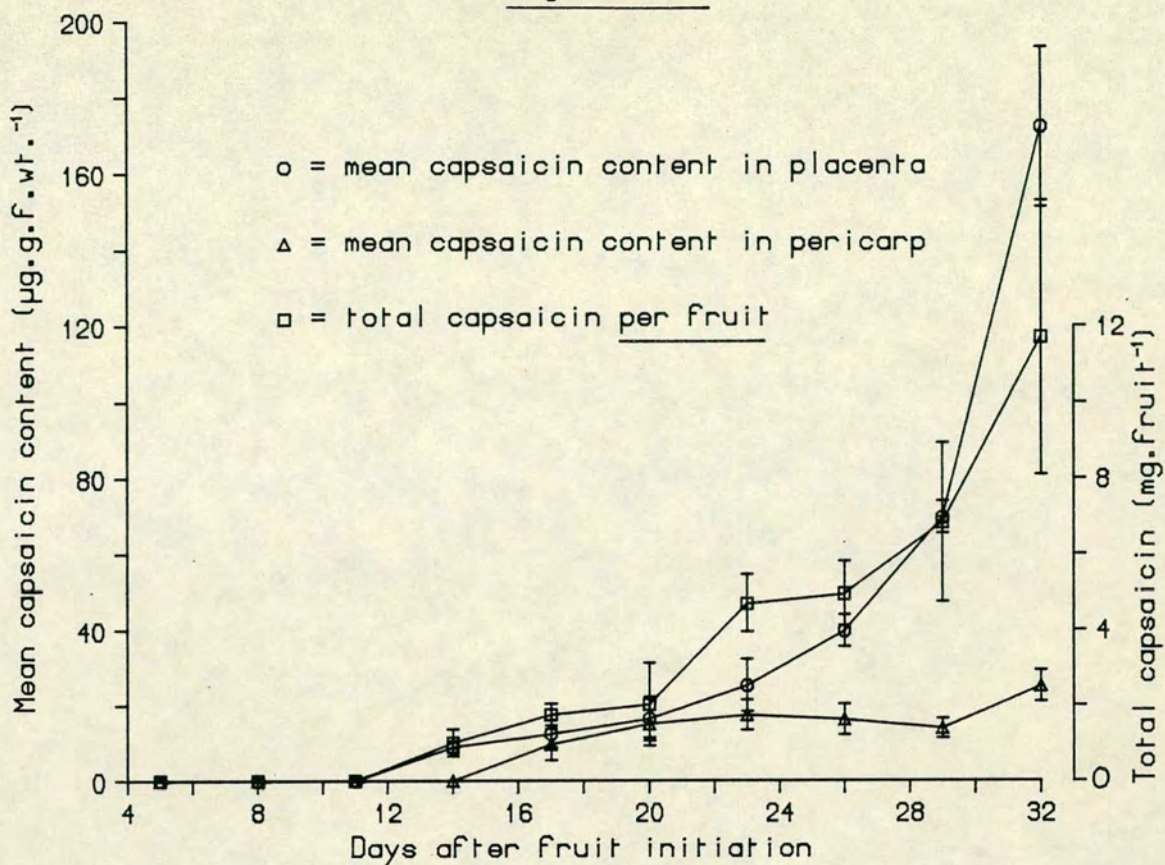
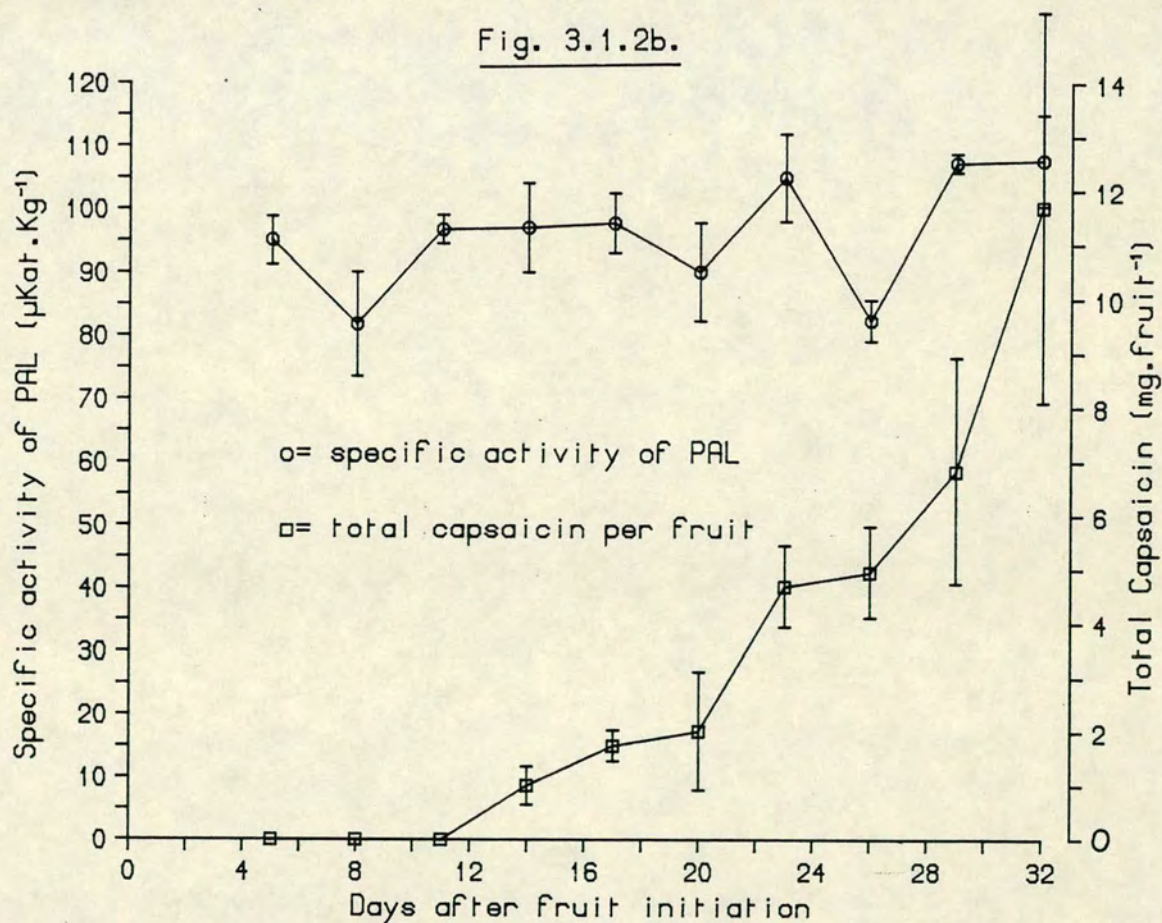


Fig. 3.1.2b.



The results presented in Fig. 3.1.2a. show the accumulation of capsaicin in whole fruit, placenta and pericarp of the fruit. No detectable levels of capsaicin were found in the seeds of the fruit and these results are not included here. Capsaicin accumulation in the whole fruit started between 10 and 14d. after fruit initiation. After day 20 there was an increase in capsaicin content, reaching *ca.* 12 mg.fruit⁻¹ at 32d. Capsaicin accumulation in the pericarp was limited and reached a maximum (*ca.* 20 µg.g.f.wt.⁻¹) 20 d. after fruit initiation. The placenta of the fruit appeared to be the major site for accumulation of capsaicin where levels reached approximately 170 µg.g.f.wt.⁻¹ 32 d. after fruit initiation.

In Fig. 3.1.2b. the total capsaicin accumulated can be seen in relation to the specific activity of PAL throughout a time course of fruit development. There was no significant change in the level of PAL activity throughout the time course (between 82 and 107 µKat. kg.⁻¹). The level of capsaicin increased from zero at day 11 to approximately 12 mg.fruit⁻¹ at day 32.

The data presented in sections 3.1.1. and 3.1.2. show that the specific activity of PAL does not correlate with the onset of capsaicin production either in the fruit (or cultured cells) of *C.frutescens*. Although this is good evidence in support of the view that PAL is not the rate-limiting enzyme for the biosynthesis of capsaicin under the conditions used, it was felt necessary to confirm this before drawing a conclusion regarding the most likely position of the rate-limiting step. The next series of experiments involving the use of the highly selective inhibitor of PAL, L-amino oxyphenyl propionic acid (AOPP), was performed to further study the possible regulatory role of PAL in the capsaicin biosynthetic pathway.

3.1.3. Use of the inhibitor AOPP to test whether or not PAL activity is limiting the biosynthesis of capsaicin *in vivo* in fruits of *C.frutescens*.

The use of enzyme inhibitors to test whether an enzyme activity is likely to be limiting the overall flux through a metabolic pathway has been reported by several workers (Rognstad, 1979., Amrhein and Godeke, 1977). The theory behind the method is described in detail by Rognstad, 1979., a short introduction identifies the main features:

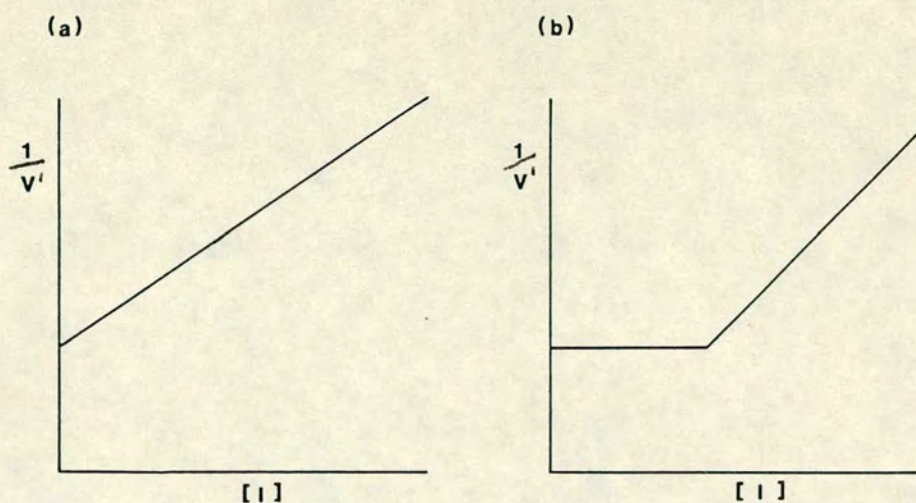
If a given enzymatic step is rate-limiting, the activity of that enzyme is not sufficient to bring the reactants and products to equilibrium. Increasing or decreasing the concentration of such an enzyme should therefore increase (or decrease) the overall flux through the pathway. If it were possible to alter the concentration of enzymes by injecting more enzyme, it would be possible to test which enzymes were rate-limiting under a given set of conditions. In general this is not feasible however, in principle it is possible with the use of highly selective inhibitors which lower the effective activity of an enzyme. Inhibitors have been used extensively to test whether a given pathway includes the enzyme on which the inhibitor acts. Some of this work has been criticized on the basis that high concentrations of inhibitor are often used. Such high levels might interfere with other aspects of metabolism and results of this nature generally need to be interpreted with care. The approach described by Rognstad, 1979., involves the use of specific inhibitors over a wide concentration range in a more subtle and refined way.

The rate equation for a competitive inhibitor may be written as follows:

$$\frac{1}{v} = \frac{K_m}{V_{\max} \cdot K_i \cdot [S]} \cdot [I] + \frac{K_m + [S]}{V_{\max} \cdot [S]}$$

where v = the flux through the enzyme. Therefore a plot of $1/V$ against $[I]$ would be a single straight line if $[S]$ is kept constant.

If V' is the overall flux through the pathway of which the enzyme on which the inhibitor acts is a part, there are 2 possible plots of $1/V'$ against $[I]$:

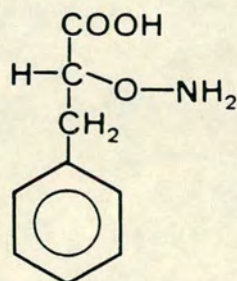


a) If the inhibitor acts on a rate-limiting enzyme, the inhibitor will decrease the overall flux at all concentrations of the inhibitor used and the plot will be a single straight line.

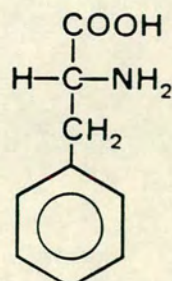
b) If the inhibitor acts on a non rate-limiting enzyme, there will be an initial null effect and a lag phase as low concentrations of inhibitor act on the excess enzyme. The inhibitor will not decrease overall flux through the pathway until a certain concentration of inhibitor is reached.

In this series of experiments, AOPP (a kind gift from Dr. Steve Fry) was used in conjunction with [^{14}C]phenylalanine in order to determine whether or not PAL is the rate-limiting step for the production of capsaicin. L-amino oxyphenyl propionic acid (AOPP), the hydroxylamine analogue of phenylalanine (Fig. 3.1.3b.) is a potent highly selective inhibitor of phenylalanine ammonia-lyase (PAL).

Fig. 3.1.3b.



L-amino oxyphenyl propionate



phenylalanine

The aim of the experiments described in section 3.1.3. is to determine whether or not PAL is likely to be a rate-limiting enzyme for the production of capsaicin in fruits of *C.frutescens*. Fruits of *C.frutescens* were used in most experiments since at a particular stage of development they are known to be producing capsaicin. Cultured cells of *C.frutescens* were used in the first experiment to test the inhibitory capacity of AOPP *in vitro*, since the spectrophotometric assay of PAL in an extract obtained from cultured cells had been optimized. Before it was possible to run the Rognstad experiment, it was necessary to carry out 3 other experiments:

- To determine the ability of AOPP to inhibit PAL in a crude cell-free extract of pepper cells.
- To determine the incubation time to be used.
- To determine the concentration range of AOPP to be used.

3.1.3.1 Measurement of the ability of AOPP to inhibit PAL activity in a cell-free extract obtained from suspended cells of *C.frutescens*.

This experiment was designed to test whether or not AOPP is effective in inhibiting PAL activity obtained from cells of *C.frutescens*. It was first necessary to establish the ability of AOPP to inhibit PAL activity before continuing with the Rognstad experiment to test the possibility that PAL might be rate-limiting for the production of capsaicin.

Suspended cells of *C.frutescens* (10 d. old) *ca.* 2.0g. were subcultured into fresh medium and left for 6 h. to allow the PAL activity within the cells to reach a measurable level. The cells were then harvested by filtration through Whatman 1. paper and extracted for PAL activity (section 2.5.1.7). The specific activity of PAL in the presence and absence of AOPP was determined using [^{14}C]phenylalanine (1 μCi . per assay tube) and measuring the incorporation of label into cinnamate. The AOPP was prepared in buffer as for the [^{14}C]phenylalanine (section 2.5.2.1) and where AOPP was omitted from the assay, an appropriate volume of buffer was added. Reaction conditions were as described in section 2.5.2.1. Following the 2 h. incubation period, the reaction mixtures were partitioned with chloroform and treated as described in section 2.6.1.2. The extract obtained was then analysed using HPLC (section 2.6.3.) and the fractions eluted from the column were counted (section 2.6.4.). Using this method it was possible to measure incorporation of the label from [^{14}C]phenylalanine into cinnamate in the presence and absence of AOPP.

From the results presented in Fig. 3.1.3.7 it can be seen that there was a significant decrease (from *ca.* 50,000 dpm. to *ca.* 10,000 dpm.) in the amount of ^{14}C label incorporated into cinnamate as the concentration of AOPP was increased from 10^{-7}M . to 10^{-5}M . Since an *in vitro* assay system was used and no label was found in any of the other intermediates of the capsaicin biosynthetic pathway, it may be concluded that AOPP is effective in inhibiting PAL activity. The inhibitor can thus be used in subsequent experiments to test whether or not PAL might be rate-limiting for the biosynthesis of capsaicin.

Fig. 3.1.3.1.

Effect of AOPP concentration on the incorporation of label from [^{14}C] phenylalanine into cinnamate in an extract from suspended cells of *C.frutescens*. Each point represents the mean of three replicates \pm standard error.

Fig. 3.1.3.2.

Incorporation of [^{14}C]phenylalanine into capsaicin over 160 min. following injection of the label into the loculus of fruits of *C.frutescens*. Each point represents the mean of three replicates \pm standard error.

Fig. 3.1.3.(1).

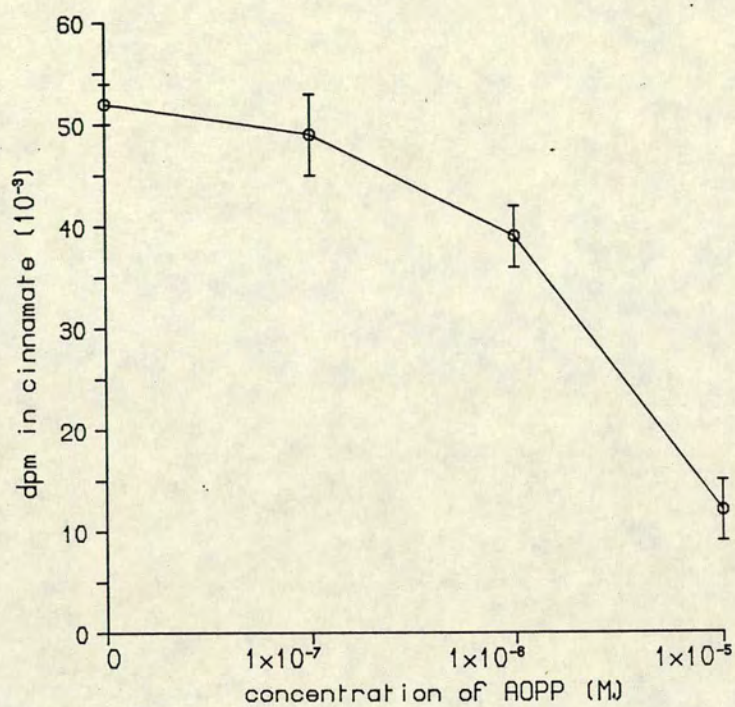
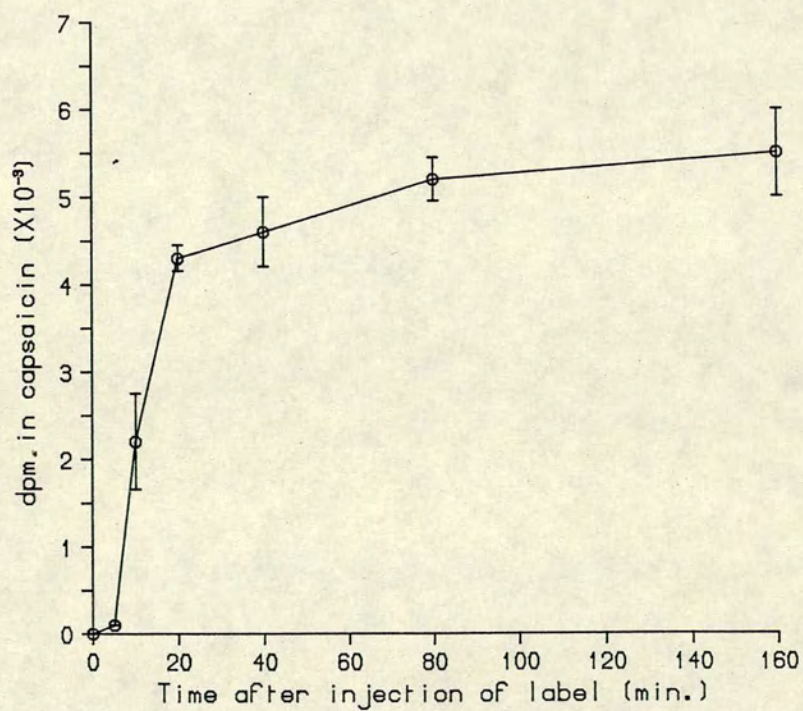


Fig. 3.1.3.(2).



3.1.3.2 Determination of the time required for measurable quantities of ^{14}C to be detected in capsaicin following injection of fruits of *C.frutescens* with [^{14}C]phenylalanine.

It is known that prolonged exposure to inhibitors reduces the selectivity of inhibition and increases the likelihood that general metabolism may be affected. It was therefore necessary to determine the minimum incubation time required to ensure the incorporation of measurable amounts of radiolabel into capsaicin.

Attached fruits of *C.frutescens* of a similar age (21 d. old) were injected with [^{14}C]phenylalanine (1 μCi . per fruit) at time=0. The fruits were harvested at various time intervals following injection of the label and extracted in methanol (section 2.6.1.1). The methanol extract was treated as described in that section and then analysed using 2D-TLC (section 2.6.2.3). On the TLC plate the capsaicin spot was located, scraped and counted (section 2.6.4.). This enabled the minimum time for incorporation of label into capsaicin to be determined and an optimum incubation time to be selected.

From the data presented in Fig. 3.1.3.2 it can be seen that ^{14}C was found in capsaicin as early as 10 min. after the injection of the fruit with [^{14}C]phenylalanine. Earlier results have shown that incorporation into capsaicin can occur as quickly as 4 min. after injection of [^{14}C]phenylalanine (Hall *et al.*, 1987). For the purposes of this experiment it was necessary to select an incubation time that would allow measurable quantities of label into capsaicin so that differences in response to varying AOPP concentrations could be detected. A one hour incubation time was selected.

It was recognized that since all the labelled substrate was likely to have been used up (after approximately 60 min.), these data may represent extent of reaction and not reaction rate.

3.1.3.3 Determination of the concentration range of AOPP which inhibits PAL activity *in vivo* in fruits of *C.frutescens*.

It has already been shown that a concentration of 10^{-5} M. AOPP will strongly inhibit PAL activity in an *in vitro* extract from cultured cells (section 3.1.3.1). This experiment was designed to determine the concentration range of AOPP over which PAL is inhibited *in vivo* in fruits.

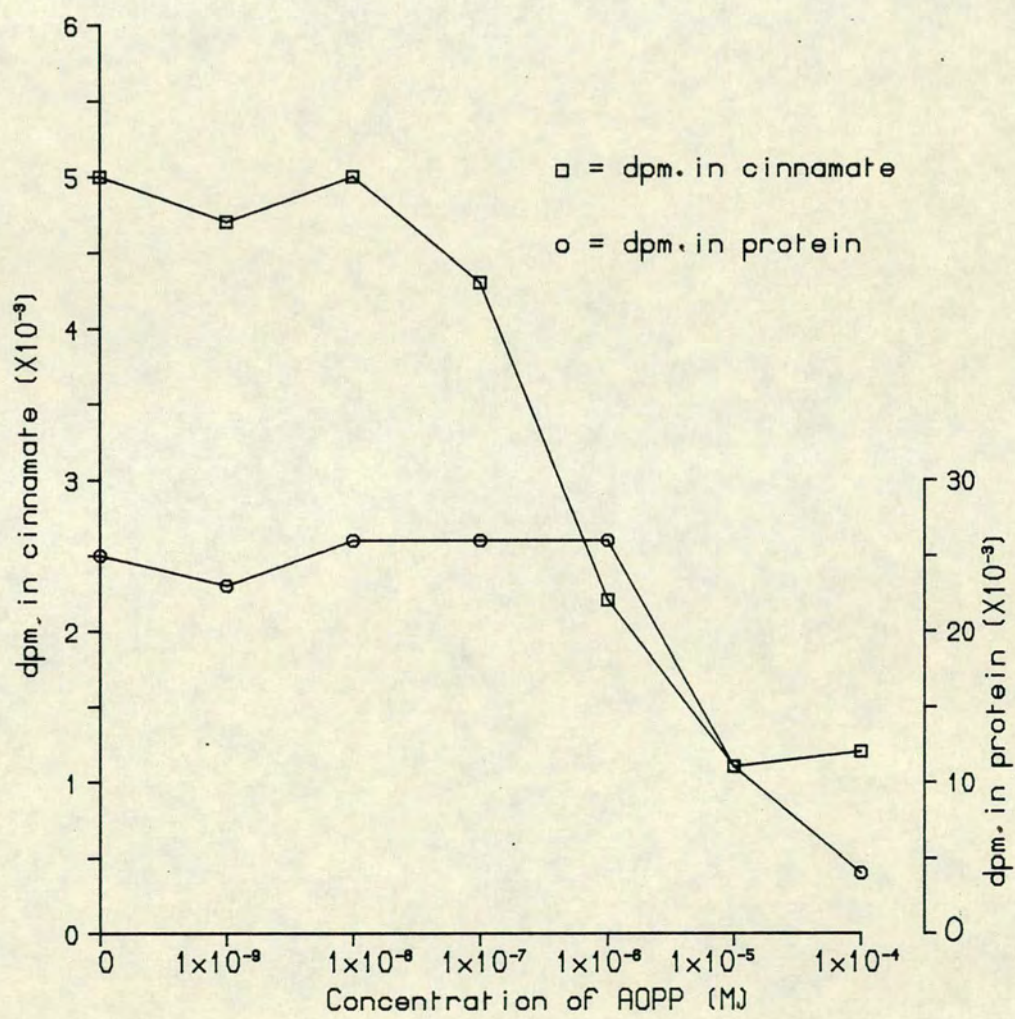
A wide concentration range of AOPP (10^{-9} to 10^{-4}) was chosen for this experiment to ensure that concentrations were used that would inhibit PAL activity *in vivo*. The fruits (age 21 d. old) were injected with [^{14}C]phenylalanine (1 μCi . per fruit) and AOPP simultaneously. After 1 h. the fruits were harvested and extracted in methanol as described in section 2.6.1.7 and the extract obtained was then analysed using HPLC (section 2.6.3.) and counted (section 2.6.4.). The amount of radiolabel in cinnamate was determined at each concentration of AOPP. It was also important to confirm that AOPP did not have a significant effect on general metabolism. This was tested by measuring the incorporation of label from [^{14}C]phenylalanine into non-covalently bound cell protein using the method of Fry, (1988).

The results presented in Fig. 3.1.3.3 show that AOPP is effective in inhibiting PAL *in vivo* at concentrations above 10^{-8}M . The amount of radiolabel incorporated into cinnamate decreased between 10^{-7} and 10^{-5} . The results in Fig. 3.1.3.3 also show a fairly constant level of incorporation of ^{14}C label from phenylalanine into protein over the AOPP concentration range 0 – 10^{-6}M . At concentrations of 10^{-5}M . and above the AOPP tended to decrease the amount of radiolabel incorporated into protein. This suggests that at concentrations of AOPP 10^{-5} and above the inhibitor is strongly affecting protein metabolism in addition to PAL activity. Concentrations of AOPP used in experiment 3.1.3.4 were selected between $5 \times 10^{-8}\text{M}$. and $1 \times 10^{-6}\text{M}$. since all these levels of AOPP inhibit PAL *in vivo* but do not appear to affect general metabolism (as indicated by the constant level of incorporation of label into protein).

Fig. 3.1.3.3.

Incorporation of radiolabel into cinnamate (squares) and protein (circles) over a range of AOPP concentrations following injection of fruits of *C.frutescens* with [¹⁴C]phenylalanine.

Fig. 3.1.3.(3).



3.1.3.4 The use of the inhibitor AOPP to test whether or not PAL is likely to be the rate-limiting enzyme for the biosynthesis of capsaicin in fruits of *C. frutescens*.

Since AOPP is known to be an effective inhibitor of PAL in fruits of *C. frutescens* over a range of concentrations (see previous experiments), this experiment was designed to test the possibility that PAL might be the rate-limiting enzyme for the production of capsaicin. Combined data obtained from sections 3.1.1., 3.1.2. and 3.1.3. can then all be used to draw some conclusion regarding the likely position of the rate-determining step for capsaicin biosynthesis.

Attached fruits (of *C. frutescens* 21 d. old) were injected simultaneously with [^{14}C]phenylalanine (1 μCi . per fruit) and AOPP (over the concentration range selected in experiment 3.1.3.3). The fruits were left for one hour and then harvested, extracted in methanol and analysed as for experiment 3.1.3.3. The amount of label into capsaicinoids was determined and the results of the experiment are presented in Fig. 3.1.3.4. The incorporation of label from [^{14}C]phenylalanine into non-covalently bound cell protein was also determined as for section 3.1.3.3.

From Fig. 3.1.3.4. it can be seen that the amount of label found in capsaicinoid was fairly constant over the concentration range of AOPP 0–0.6 μM . At a concentration of 0.8 μM , AOPP and above there was a very large decrease in the amount of label found in capsaicinoid. Over the concentration range of AOPP used PAL is known to be inhibited (determined in experiment

3.1.3.3) and yet there was no decrease in the extent of incorporation into capsaicin until a concentration of 0.8 μM AOPP was reached. If these results are plotted as shown in Fig. 3.1.3.5. a graph of the form presented was obtained. This bi-phasic plot demonstrates the null effect of the inhibitor on the extent of incorporation into capsaicinoids at low concentrations and suggests that an enzyme other than PAL is limiting incorporation of the label into capsaicin. The second plot in Fig. 3.1.3.4. shows that there was no change in the level of incorporation of ^{14}C into protein over the AOPP concentration used. This is a good indication that metabolic processes other than PAL activity were not severely altered in the presence of AOPP.

Fig. 3.1.3.4.

Incorporation of radiolabel into capsaicinoids (squares) and protein (circles) over a concentration range of AOPP known to inhibit PAL, following injection of fruits of *C.frutescens* with [^{14}C]phenylalanine.

Fig. 3.1.3.5.

"Rognstad Plot" of the data presented in Fig. 3.1.3.4. Effect of AOPP on the incorporation of radiolabel into capsaicinoids following injection of fruits of *C.frutescens* with [^{14}C]phenylalanine.

Fig. 3.1.3. (4).

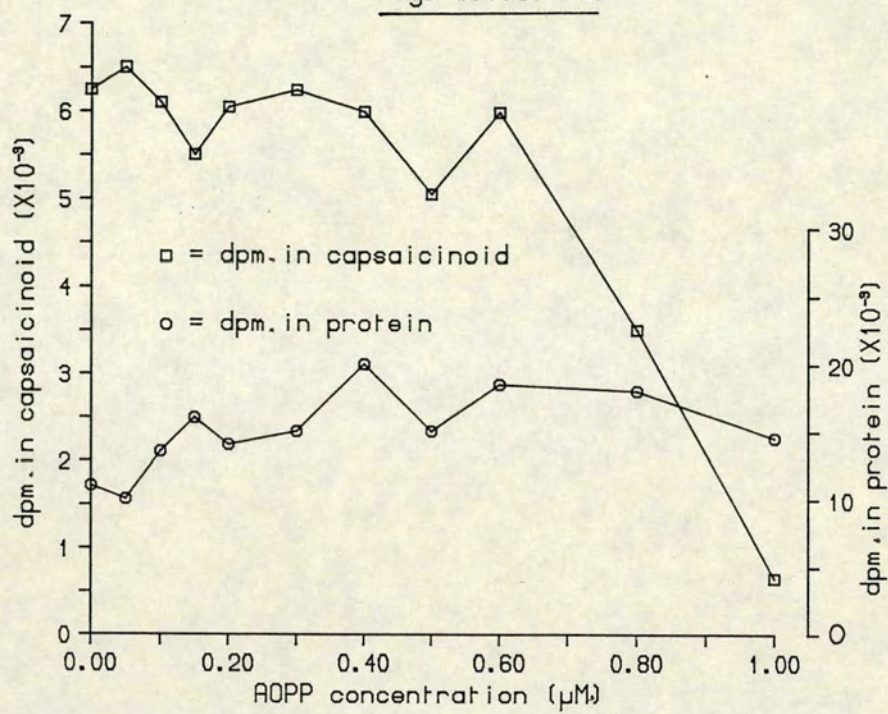
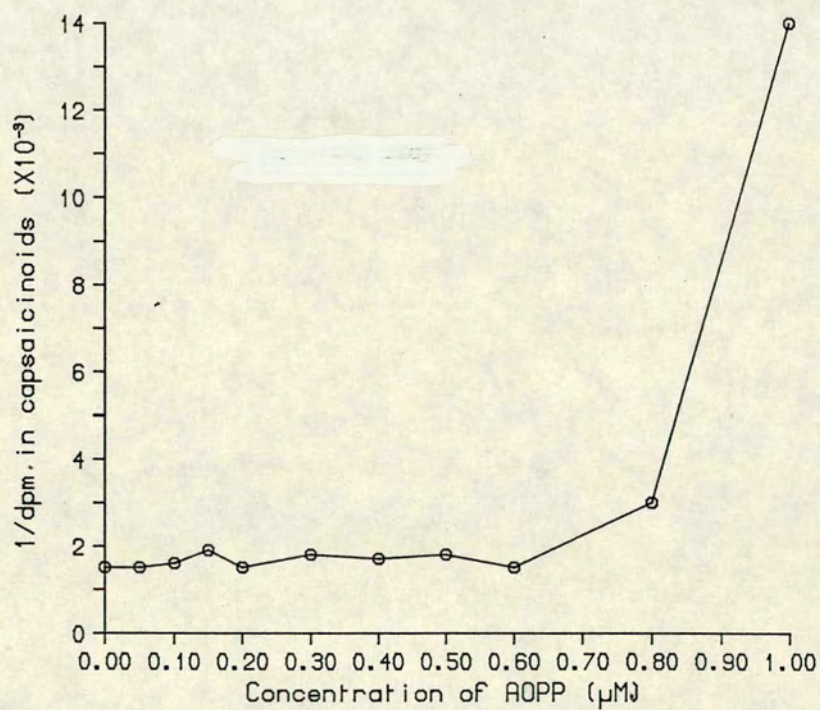


Fig. 3.1.3. (5).



It is recognized that there are several limitations associated with the technique used in section 3.1.3.. Firstly the theory behind the method is somewhat simplistic in the assumptions made regarding the kinetics of competitive inhibition. It is unlikely that the simple kinetics of competitive inhibition of an isolated enzyme operate in the intact cell, and it is realized that the actual situation in the cell would be far more complex. It was also recognized that inhibitors may affect areas of metabolism other than the specific reaction of interest. This problem was overcome to a certain extent by measuring incorporation of [^{14}C] label into protein. As there was no change in the level of incorporation into protein over the concentration range of AOPP used (Fig. 3.1.3.4), it is probable that the effects observed in response to AOPP can be attributed to the inhibition of PAL by the inhibitor. Whilst this does not confirm that all areas of metabolism remained unaffected by the AOPP, measurement of protein metabolism is a good indicator of the general metabolic state of cells. It was also noted that AOPP is a competitive inhibitor and as such it is important that inhibitor and [^{14}C]phenylalanine reach the same site in the cells. This problem was overcome to some extent by injecting the inhibitor and substrate simultaneously. Although little is known of the uptake properties of AOPP, these data are in agreement with other data (Amrhein and Godeke, 1977; Amrhein and Zenk, 1977) suggesting that AOPP is taken up quite readily by cells.

The evidence presented in section 3.1.3. suggests that phenylalanine ammonia-lyase ^{may not be} the rate-limiting enzyme for the production of capsaicin under the conditions used. This is in agreement with the data obtained in sections 3.1.1. and 3.1.2. where it was apparent that there was no significant increase in the specific activity of PAL (or C,4-H in the cultured cell system) under conditions of capsaicin biosynthesis in the fruit or in cultured cells.

Results to date have shown that limitations to capsaicin accumulation occur, most probably in the latter part of the biosynthetic pathway. The data presented in section 3.1. show that PAL and C,4-H do not appear to have rate-limiting roles for the production of capsaicin under the conditions used and evidence from other sources supports this view. Subsequent work concentrates on the terminal reaction sequence catalysed by the capsaicinoid-synthesizing complex (capsaicin synthase), which catalyses the condensation of vanillylamine and iso-type fatty acids to form capsaicinoids.

Part 3.2. Uptake and subsequent conversion of [³H] vanillylamine into cells of *C.frutescens*.

In this section attention is focussed on measuring the uptake, conversion and incorporation of [³H]vanillylamine *in vivo* in fruits and cultured cells of *C.frutescens* to: a) confirm the presence of capsaicin synthase and b) test the suitability of [³H]vanillylamine as an *in vitro* substrate for the study of capsaicin synthase.

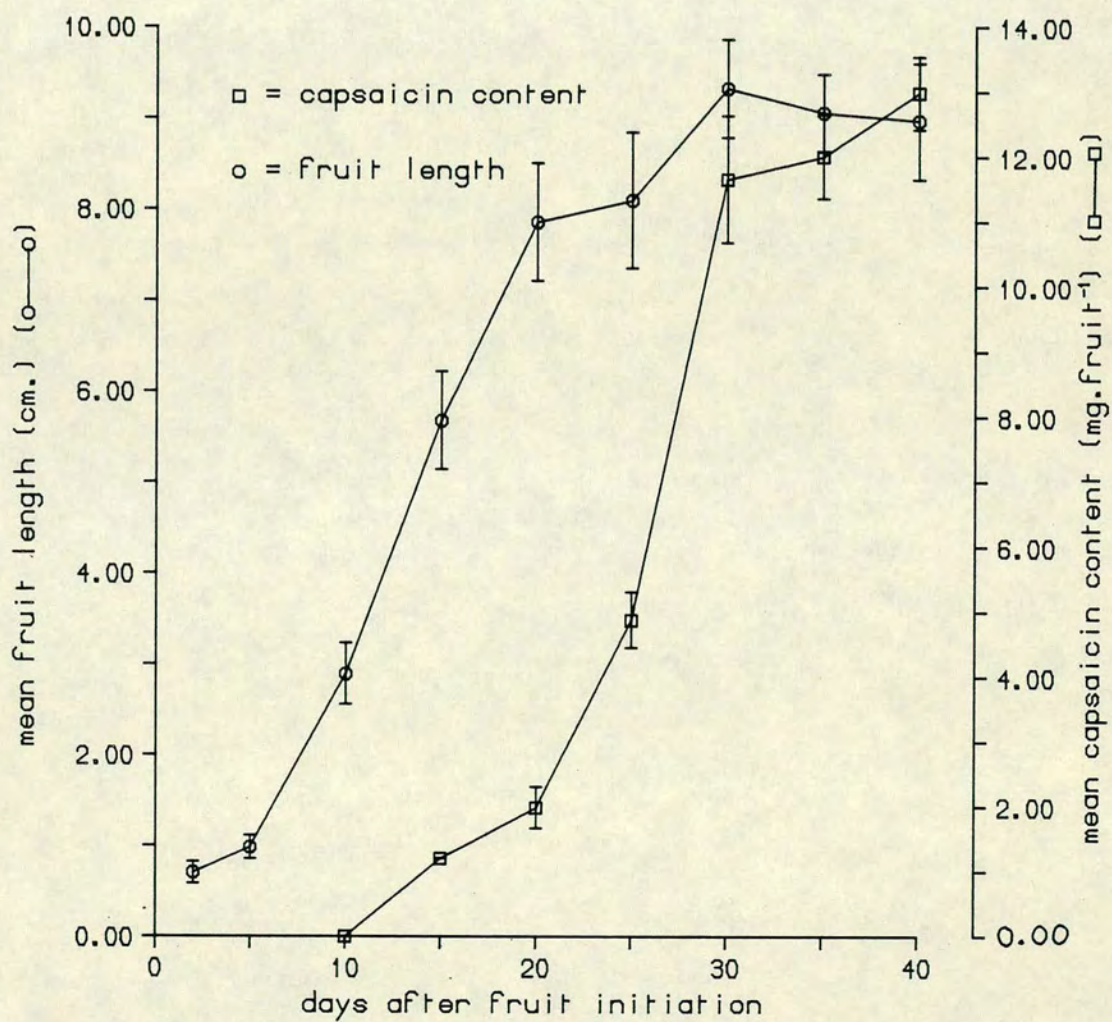
3.2.1. Measurement of the uptake and incorporation of radiolabel from [³H]vanillylamine into phenylpropanoids and capsaicinoids in fruits of *C.frutescens*.

This experiment was designed to measure the uptake and incorporation of radiolabelled vanillylamine in attached fruits of *C.frutescens*. Prior to this, a rigorous growth analysis of fruits had been completed and the correlation between fruit age, fruit length and capsaicin accumulation are presented in Fig.3.2.1. This graph was used to pre-select fruits at an age where there was a maximum chance of capsaicin synthase activity (*ca.* 20–25 d.). The substrate [³H]vanillylamine was prepared in buffers of different pH (range 8.0–9.5). This pH range was selected because the pK_i for primary amine groups is usually in the region of pH 9.0–9.3. Each fruit (25 d. old) was injected directly into the loculus with the radiolabel at a particular pH (*ca.* 2 μCi. per fruit) as described in section 2.4.2. At a pre-selected time following injection of the radiolabel, the fruits were harvested, cut open longitudinally and washed twice with the appropriate buffer to remove exogenous vanillylamine. The fruits were then extracted in methanol as described in section 2.6.1.7 and the extracts were analysed for incorporated label using 2D-TLC (section 2.6.2.3) and liquid scintillation counting (section 2.6.4.).

Fig. 3.2.1.

The relationship between fruit length, mean capsaicin content and fruit age in *C.frutescens*.

Fig. 3.2.1.



The results presented in Table 3.2.1a. show the distribution of radiolabel in the various fractions. It can be seen from column 1. that most of the label was not taken up by the cells and remains as unchanged [^3H]vanillylamine in the washings. The distribution of radiolabel in the methanol soluble fraction (column 2.) varied considerably in response to pH. One hour after injection of the radiolabel there was a significant uptake of vanillylamine at pH 9.0 and 9.5 but no radiolabel was found in the methanol fraction at pH 8.0 and 8.5. Following a 24h. incubation with the radiolabelled substrate, a similar pattern emerged. Radiolabel was only detected in the methanol fractions at pH 9.0 and 9.5. The maximum uptake into the cells was 23% after a 24h. incubation with the label at pH 9.5.

The methanol fractions which contain the phenylpropanoids and capsaicinoids of interest were highly radioactive and were analysed in detail. Table 3.2.1b. shows the total amount of radiolabel found in each of the phenylpropanoids and in capsaicin (dpm, g.f.wt. $^{-1}$). Radiolabel was not detected in any of the early intermediates of the pathway (cinnamate to caffeate) and only very low levels of radioactivity were detected in ferulate after 1h. but not after 24h. A very fast back reaction occurred converting vanillylamine to vanillin and high levels of radioactivity were found in vanillin at both pHs and all incubation times, however, a large quantity of the radiolabel remained as vanillylamine. Following a 1h. incubation with the radiolabel no incorporation into capsaicinoids was detected at pH 9.0 and 9.5. Following a 24h. incubation, incorporation into capsaicinoids was detected and a maximum level of 1,020 dpm. incorporated at pH 9.5. Generally total uptake was increased at the higher pH and this was reflected by higher levels of incorporation into vanillin and capsaicinoids at these pHs.

The uptake of tritiated vanillylamine into fruits of *C.frutescens* was shown to be greatest at pH 9.5. Conversion of radiolabel into capsaicinoids was demonstrated and it can be concluded that fruits of *C.frutescens* contain capsaicin synthase activity.

Table 3.2.1a.

The distribution of radiolabel in fractions obtained using the procedure outlined in section 3.2.1.

time after injection(h.)	distribution of radiolabel* (as % of total supplied)				% recovery
	(A)	(B)**	(C)	(D)	
1	8.0	87	0	2.0	89.0
	8.5	89	0	2.0	91.0
	9.0	92	16	1.5	109.5
	9.5	78	21	3.4	102.4
24	8.0	86	0	2.1	88.1
	8.5	92	2	3.6	97.6
	9.0	65	18	2.2	85.2
	9.5	69	23	5.1	97.1

(A) = pH of injection buffer.

(B) = washings.

(C) = methanol soluble fraction.

(D) = methanol insoluble fraction.

* All figures calculated on the basis of % label injected which was the same in all fruits.

** Washings= radiolabel found in the buffer fraction which was used to wash the cells prior to methanol extraction.

Table 3.2.1b.

The distribution of radiolabel in the capsaicinoids and phenylpropanoid intermediates of the capsaicin biosynthetic pathway.

	total dpm into capsaicinoid and phenylpropanoid compounds			
	time=1h.		time=24h.	
	pH 9.0	pH 9.5	pH 9.0	pH 9.5
cinnamate	0	0	0	0
coumarate	0	0	0	0
caffeate	0	0	0	0
ferulate	32	34	0	0
vanillin	1,255	2,961	1,049	5,964
vanillylamine	4,965	10,063	4,821	7,321
capsaicinoid	0	0	365	1,020

The results presented in section 3.2.1. showed that there was an optimal pH for the uptake of [³H]vanillylamine and that conversion of the radiolabelled substrate into capsaicinoids and vanillin took place (under certain conditions) in the fruit. The capsaicin synthase enzyme must therefore be present and active in the fruit sample examined.

3.2.2. Measurement of the uptake and incorporation of radiolabel from [³H]vanillylamine into phenylpropanoids and capsaicinoids in cultured cells of *C.frutescens*.

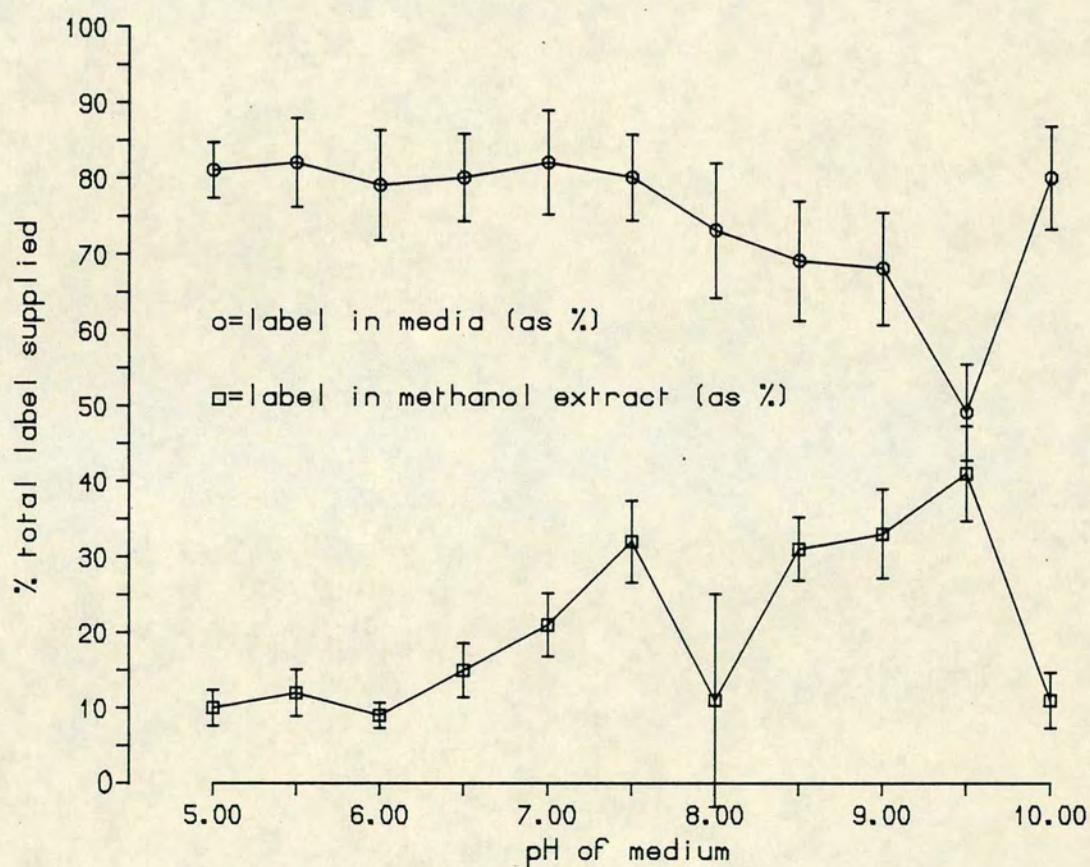
The present experiment was designed to examine the uptake and subsequent conversion of [³H]vanillylamine *in vivo* in suspended cells of *C.frutescens* over a range of pH treatments to test if capsaicin synthase activity can be detected in cultured cells.

Suspended cells of *C.frutescens* were subcultured from one flask into 250ml. Erlenmeyer flasks (*ca.* 2g. per flask) containing SH medium buffered to a selected pH with Universal buffer mixture (for composition of buffer mixture see Biochemist's Handbook). In addition, the flasks contained [³H]vanillylamine (*ca.* 1 μ Ci. per flask) which had been filter-sterilized prior to use (section 2.2.2.4). The cells were incubated with the radiolabel under normal growth conditions for 2h. after which time they were harvested by filtration through Whatman 1. paper. The cells were washed twice with buffer of the appropriate pH and then extracted in methanol as described in section 2.6.1.7. Analysis was performed using HPLC (section 2.6.3.) and liquid scintillation counting (section 2.6.4.). The media were extracted in chloroform (section 2.6.1.2) and analysed using the same techniques as for the methanol extracts. A parallel non-radioactive experiment confirmed that the pH treatments were maintained throughout the 2h. period. On average, cell viability had declined *ca.* 7% by the end of the 2h. treatment.

Fig. 3.2.2.

Distribution of radiolabel in the media and in the methanolic extracts of cells following incubation of suspended cells of *C.frutescens* with [^3H]vanillylamine.

Fig. 3.2.2.



The results presented in Fig.3.2.2. show the amount of radiolabel taken up into the cells and the amount which remained in the media as percentages of the total radiolabel supplied. The amount of radiolabel which remained in the media was relatively constant over the pH range 5.0–9.0. At pH 9.5, there was a significant decrease (from 80% to 50%) in the amount of radiolabel recovered in the media. At pH 10.0 the effect was reversed and more than 80% was recovered in the media. In comparison, the methanol extracts (with the exception of pH 8.0) showed a steady increase in radiolabel content over the pH range 6.0–9.5 (from 10% to 40%). At pH 10.0 the level of radiolabel in the cells had fallen to *ca.* 10%. The result obtained at pH 8.0 was due to one low replicate. The data presented in Table 3.2.2. show the distribution of radiolabel in capsaicinoids and vanillin at the selected pH treatments in the methanol extracts. The level of incorporation into vanillin varied between 3 and 32% and was highest at pH 9.5. Incorporation into capsaicinoids was not detected at any pH. Radiolabel which remained in the media was confirmed to be present as unchanged [^3H]vanillylamine.

Table 3.2.2.

The distribution of tritium label in vanillin and capsaicin in cells of *C.frutescens* incubated in media of differing pH.

	distribution of radiolabel (% of total supplied)										
pH of media	5	5.5	6	6.5	7	7.5	8	8.5	9	9.5	10
label in vanillin	8	6	5	3	10	12	10	28	22	32	10
label in capsaicin	0	0	0	0	0	0	0	0	0	0	0

Although uptake of [^3H]vanillylamine was shown to occur over a wide pH range in suspended cells of *C.frutescens*, no detectable levels of incorporation into capsaicinoids were found.

As was shown in experiment 3.2.2. the pH optimum for uptake of [^3H]vanillylamine into suspended cells was *ca.* pH 9.5. No label was found in capsaicinoids even at this pH and it was decided that an investigation into the uptake of [^3H]vanillylamine in suspended cells of different ages would perhaps show differences in the pattern of radiolabelling.

3.2.3. Uptake and incorporation of label from [³H]vanillylamine into components of the capsaicin biosynthetic pathway in cultured cells at different stages of the culture cycle.

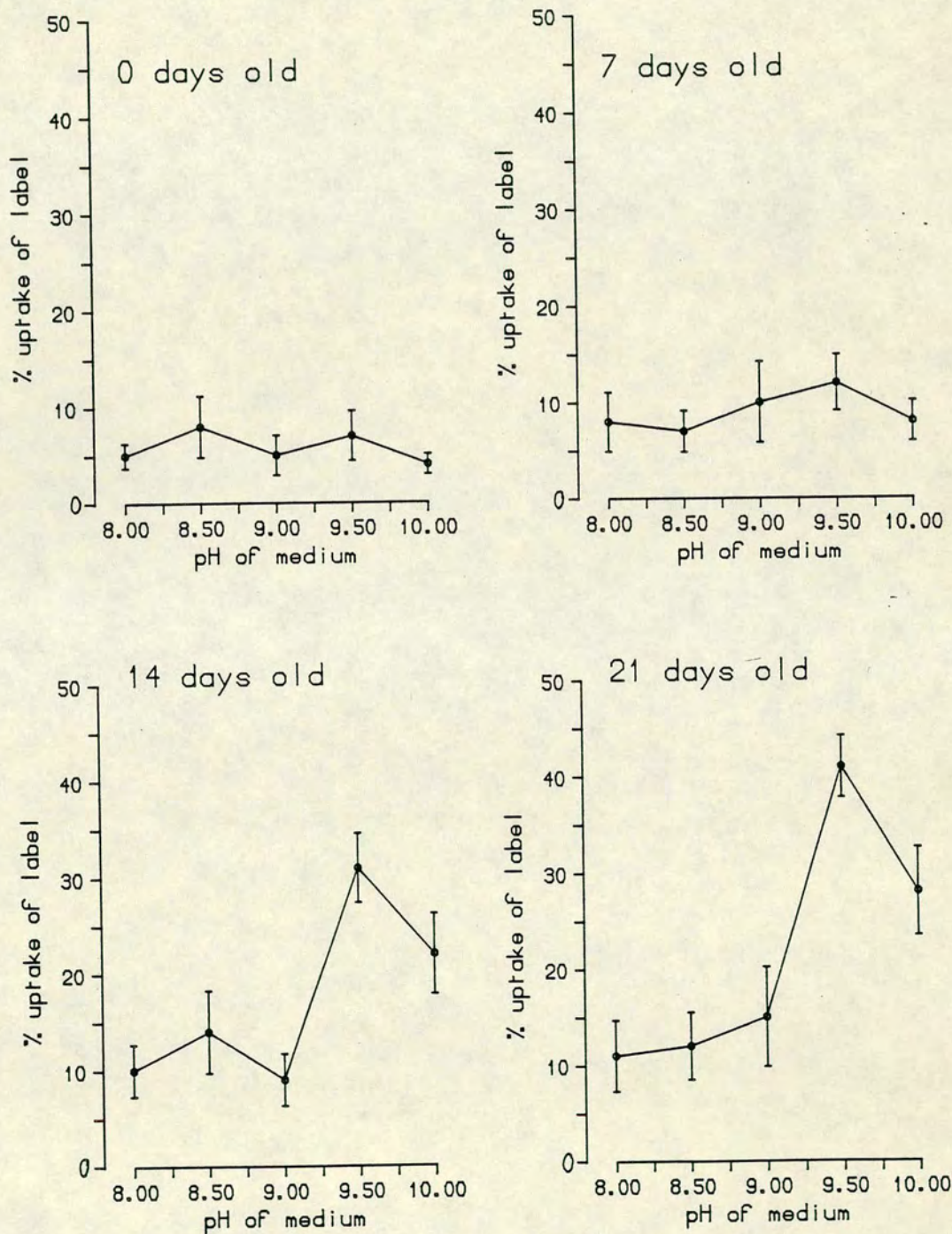
This experiment was designed to measure the uptake and incorporation of radiolabel from [³H]vanillylamine into suspended cells of *C.frutescens* of selected ages.

Suspended cells were subcultured from one flask into 250ml. Erlenmeyer flasks containing 50ml. of SH media buffered to a selected pH with Universal buffer mixture (*ca.* 2g. per flask). This subculture procedure was repeated for each of the selected age treatments (0, 7, 14, and 21 d. old). In addition the flasks contained [³H]vanillylamine (*ca.* 1 μ Ci. per flask) which had been filter-sterilized (section 2.2.2.4) prior to addition. The cells were incubated with the radiolabel under normal growth conditions (section 2.2.3.1) for exactly 2h. after which they were harvested by filtration through Whatman 1. paper. The cells were washed twice with buffer of an appropriate pH and extracted in methanol as described in section 2.6.1.1. Analysis was performed using HPLC (section 2.6.3. and liquid scintillation counting (section 2.6.4.).

The results presented in Fig.3.2.3. show the % uptake of radiolabel into the cells. Cells at 0 d. accumulated only low levels of the label (<10% of the total supplied) and no significant response to pH was detected. At 7 d. there was more radiolabel taken up by the cells in total than at day 0, but there was still no significant response to pH. Cells at 14 d. showed a marked pH optimum for the uptake of [³H] vanillylamine, the maximum occurred at *ca.* pH 9.5 and was about 32% of the total radiolabel supplied. Once again the cumulative uptake of radiolabel was greater in 14 d. old cells than in 0 or 7 d. old cells. The pH optimum was more pronounced in cells that were 21 d. old and a maximum uptake over the 2h. period of *ca.* 41% was measured in these cells. HPLC analysis of the chloroform extracts of media showed that radiolabel that remained in the media was in the form of [³H]vanillylamine.

Fig. 3.2.3.

Uptake of radiolabel into cells of *C. frutescens* over a range of different pH treatments following incubation of cells of various ages (0, 7, 14, and 21d.) with [^3H]vanillylamine.



The results presented in Table 3.2.3a. show the levels of incorporation of radiolabel into vanillin in cells of different ages over a selected pH range. No significant incorporation was measured at pH 8.0 or 8.5 most probably because very little radiolabel was taken up at these pH treatments. At pH 9.0 there were low levels of incorporation into vanillin and the level of incorporation increased with increasing cell age (probably a result of greater availability of vanillylamine due to increased uptake). Maximum incorporation into vanillin was measured in 21 d. old cells at pH 9.5 (118 cpm.g.f.wt.⁻¹). The pattern of incorporation at pH 10.0 was the same^a as for pH 9.5 but the levels were very much lower.

In Table 3.2.3.b. incorporation into capsaicinoids is shown. No incorporation was detected over the entire pH range in 0, 7 and 14 d. old cells, suggesting that vanillylamine availability is not the only controlling element since it was known to be taken up into the cells at pH 9.5. In 21 d. old cells there was no incorporation into capsaicinoids at pH 8.0 and 8.5 but at pH 9.0, 9.5 and 10.0 there was significant incorporation into capsaicinoids. A maximum level of incorporation was measured in 21 d. old cells at pH 9.5 (120 cpm.g.f.wt. ⁻¹).

Table 3.2.3a.

Incorporation of radiolabel into vanillin in suspended cells of *C.frutescens* of selected ages over a range of pH treatments.

Age(d)	incorporation of radiolabel into vanillin (cpm.g.f.wt. ⁻¹)			
	0	7	14	21
pH				
8.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
8.5	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 0.0	10.0 ± 5.2
9.0	9.7 ± 0.3	8.7 ± 0.3	56.1 ± 1.7	79.0 ± 9.3
9.5	53.3 ± 2.7	43.7 ± 2.2	100.3 ± 2.9	118.3 ± 4.5
10.0	22.7 ± 2.7	7.7 ± 2.6	46.7 ± 4.1	36.0 ± 2.3

Table 3.2.3b.

Incorporation of radiolabel into capsaicin in suspended cells of *C.frutescens* of selected ages over a range of pH treatments.

Age(d.)	incorporation of radiolabel into capsaicin (cpm.g.f.wt. ⁻¹)			
	0	7	14	21
8.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
8.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
9.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	49.6 ± 5.3
9.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	120.3 ± 9.6
10.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	31.3 ± 5.4

Uptake and incorporation of [^3H]vanillylamine into capsaicinoids was shown to occur in suspended cells of *C.frutescens* in the late stages of the culture cycle. From the evidence provided, it would appear that capsaicin synthase activity is present in suspended cells at a particular stage of the culture cycle.

The evidence provided in Part 3.2. demonstrates the uptake and subsequent conversion of [^3H]vanillylamine into capsaicinoids in cells of *C.frutescens* under certain conditions. The data support the idea that capsaicin synthase activity is present in these cells at a particular stage of fruit/culture development. Furthermore, since radiolabel is incorporated from vanillylamine into capsaicinoids *in vivo*, it is most likely that [^3H]vanillylamine is suitable for use in an *in vitro* assay system to measure capsaicin synthase activity. The results presented in the next part show the development of an assay for capsaicin synthase.

The evidence presented in section 3.1. is supportive of the idea that phenylalanine ammonia-lyase and cinnamate 4-hydroxylase are not the rate-limiting enzymes for the production of capsaicin under the conditions studied. Other work (Yeoman *et al.*, 1989), has shown that a limitation to capsaicin accumulation occurs at or near the end of the pathway. This would tend to suggest that the ability of the fruit to produce capsaicin relates to an enzyme activity near the end of the pathway, which is present at a particular stage of development.

The work described in section 3.2. demonstrated that incorporation of [^3H]vanillylamine into capsaicin occurs *in vivo* under certain conditions in both the fruit and the cell suspension culture systems. There are limitations to the usefulness of such experiments and in any full enzymological study, a reliable and sensitive assay procedure is required. The present section describes the development of a suitable assay procedure for the terminal reaction catalysed by the capsaicinoid synthesizing complex (which will be referred to as capsaicin synthase from now on).

Part 3.3. Development of an *in vitro* assay procedure for capsaicin synthase.

Although cultured cells of *C.frutescens* can produce capsaicin in significant quantities, the levels of enzyme activity are usually much lower than in the fruits. In addition, the problems of growing sufficient biomass for extensive experimentation make it difficult to develop an assay for a new enzyme using cultured cells. For these reasons it was decided to start the *in vitro* work using the fruit of *C.frutescens* which are easy to grow, contain significant quantities of the enzyme and produce capsaicin in substantial amounts.

3.3.1. Measurement of the incorporation of radiolabel from [³H] vanillylamine into capsaicinoids in an *in vitro* assay system using a crude cell-free extract from fruits of *C.frutescens*.

In this experiment an attempt was made to measure the incorporation of radiolabel from [³H]vanillylamine into capsaicin using a crude cell-free extract of fruits. A crude cell-free extract was used initially to ensure, as far as possible, that no essential cofactors were removed and to maintain the enzyme in an active form.

Fruits of *C.frutescens* of all ages were selected, harvested and weighed (section 2.1.). The fruits were extracted for capsaicin synthase activity as described in section 2.5.1.2 with the alterations described here: potassium phosphate buffer (0.1M) pH 8.0 was used containing all the components listed in section 2.5.1.2 except PMSF. Sephadex chromatography employing PD-10 columns was not used. The supernatant obtained from the 1000xg. spin was used directly for the assay of capsaicin synthase. Soluble protein content of the extract was determined using the method of Bradford (1976) (section 2.5.2.). Capsaicin synthase was assayed as described in section 2.5.2.2 [system 2]. The reaction mixtures were extracted in chloroform to terminate the reaction, this effectively separated the substrate vanillylamine (which remained in the aqueous fraction) from the product capsaicin (extracted into the chloroform) (section 2.6.1.2). Subsequent analysis was by HPLC (section 2.6.3.), fractions were collected (section 2.6.3.5) and counted (section 2.6.4.) using a liquid scintillation procedure (section 2.6.4.).

The time course of incorporation into chloroform-soluble compounds can be seen in Fig. 3.3.1a. The amount of radiolabel recovered in the chloroform

fraction increased with time, suggesting the enzymic formation of one or more chloroform-soluble compounds. The increased incorporation was MORE THAN accounted for by an increase in the formation of radiolabelled vanillin (at least during the first 20 min. of the reaction). The vanillylamine deaminase reaction appeared to follow normal single substrate kinetics as indicated by the graph obtained in Fig. 3.3.1a. where a plot of incorporation into vanillin against time shows typical saturation. No significant level of incorporation into capsaicin was measured over the duration of the time course. Fig. 3.3.1b. is a typical HPLC trace of a chloroform extract and in Fig. 3.3.1c. the distribution of radiolabel in the fractions eluted from the column is shown. Using this technique the incorporation into a particular compound was determined with a reasonable degree of specificity. The amount of radiolabel which remained in the aqueous fraction was found to be constant with time (results not shown here), probably because the % conversion into chloroform-soluble compounds was so small compared with the total radiolabel supplied.

Fig. 3.3.1a.

Incorporation of radiolabel from [³H]vanillylamine into chloroform- soluble compounds (squares), vanillin (diamonds) and capsaicin (circles) over a time course in an *in vitro* assay system.

Fig. 3.3.1a.

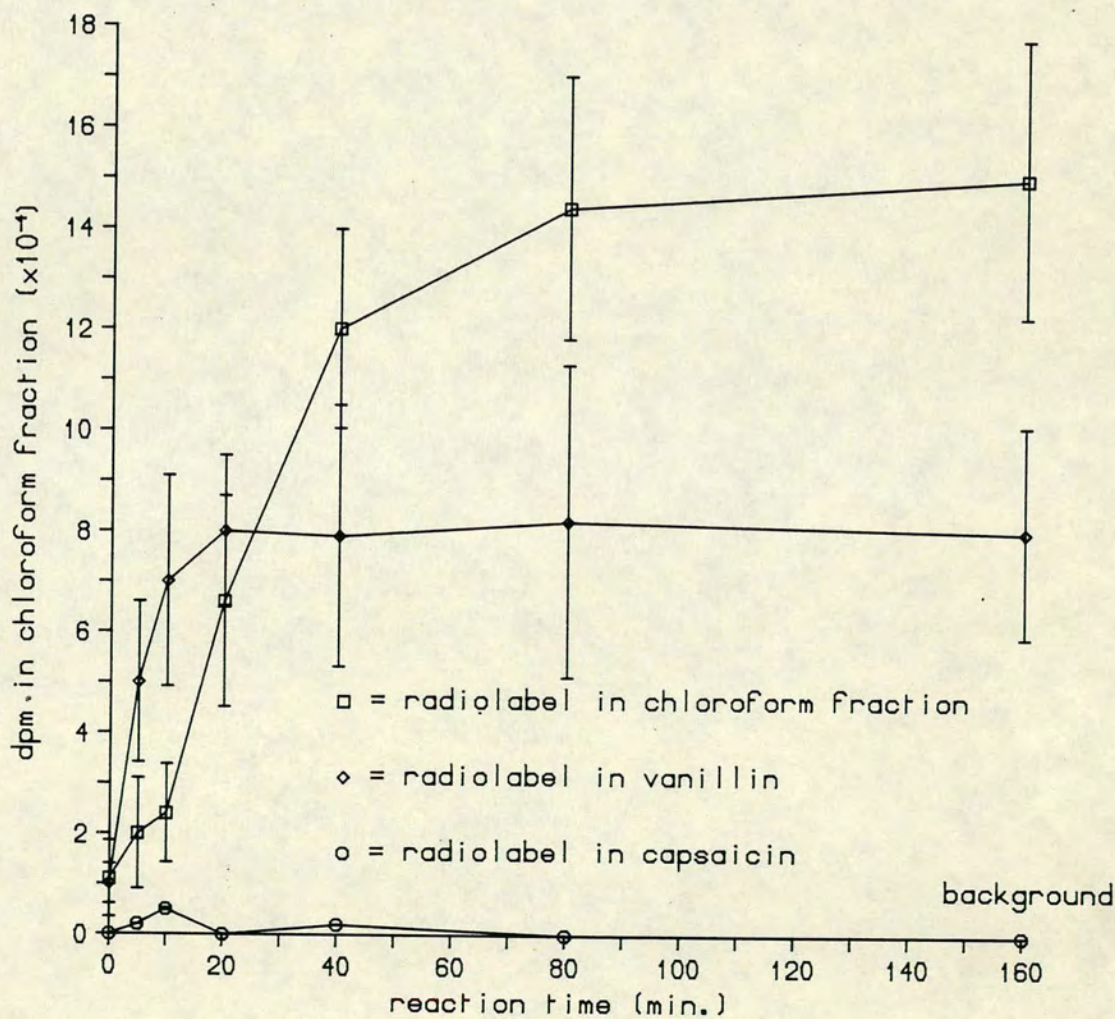


Fig. 3.3.1b.

A typical HPLC trace of a chloroform extract of reaction mixture showing the presence of vanillylamine (5.40 min.), vanillin (10.56 min.) and the capsaicinoids (22.38 and 23.17 min.).

Fig. 3.3.1c.

Levels of radioactivity in fractions eluted from the column. These data were obtained from the sample shown in Fig. 3.3.1b. and can thus be used to locate radiolabel in certain known compounds with a reasonable degree of specificity.

Fig. 3.3.1b.

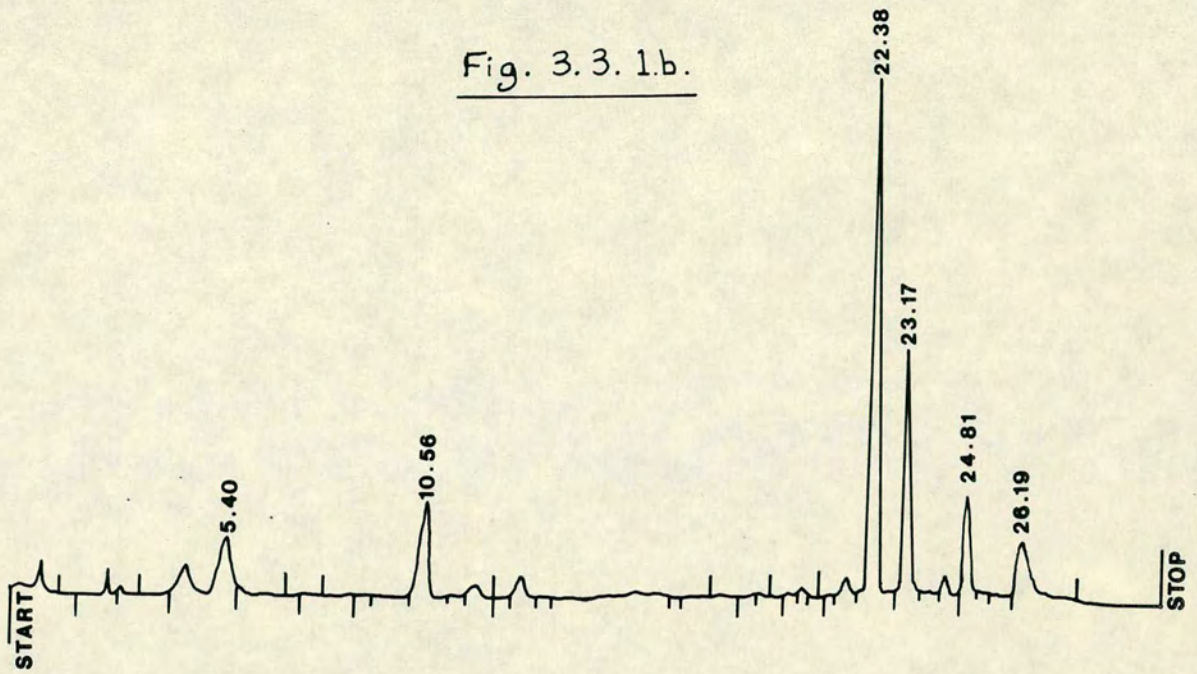
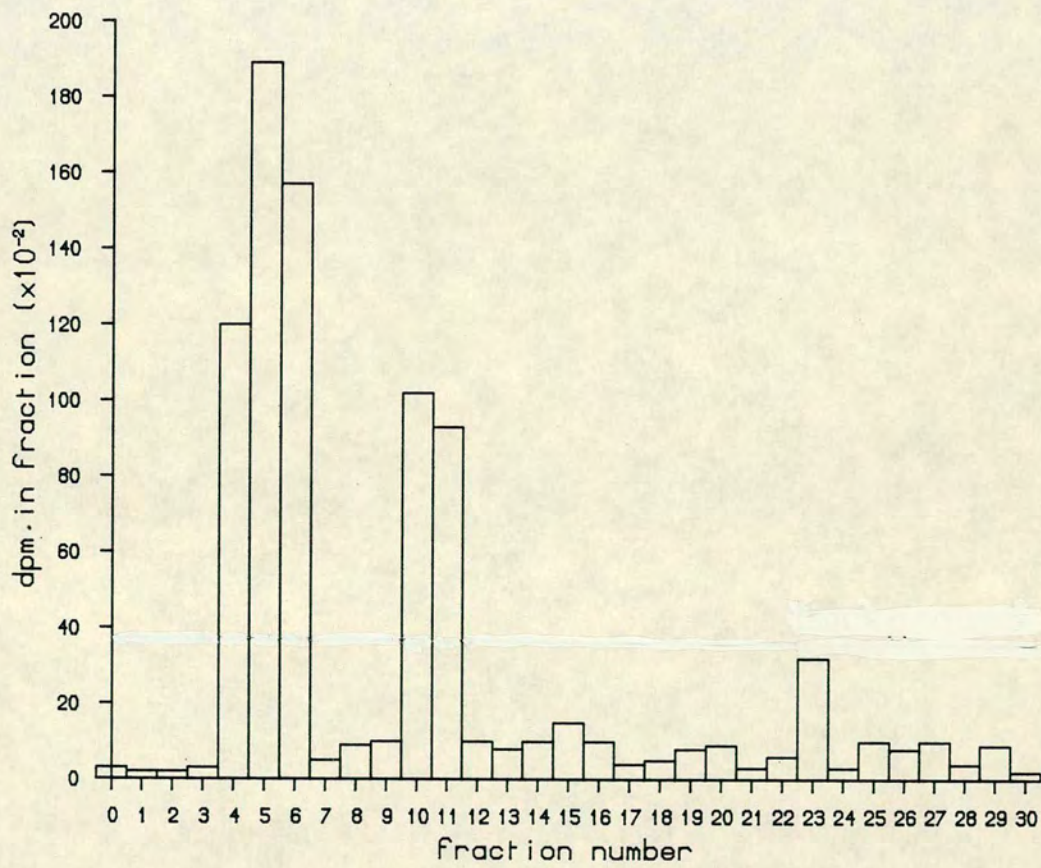


Fig. 3.3.1c.



Work in this and other laboratories has shown the presence of water-soluble phenolic esters and glycosides in plant extracts (Nicoletti *et al.*, 1988; Sukrasno, unpublished results). These conjugates are in some cases produced in vast amounts. If compounds such as these were produced in the assay of capsaicin synthase, they would not be detected in the chloroform fraction, it is therefore necessary to confirm the presence or absence of these conjugates by analysis of the aqueous fractions left after chloroform extraction. The aqueous fraction from the 40 min. reaction tube was chosen for analysis, since after 40 min. there was no further (increase in the level of) incorporation.

The aqueous fraction was clarified by centrifugation at 2000xg. for 10 min. to pellet the protein precipitated by the chloroform extraction procedure. The pellet was counted and found to contain only trace amounts of radiolabel, over 96% of the radiolabel remained in the supernatant. The supernatant was diluted to 10ml with reaction buffer and then divided in two. The first aliquot was extracted in chloroform as described in section 2.6.1.2, this served as a control extract against which the hydrolysed extracts could be compared. The second aliquot was hydrolysed with an equal volume of 1N. NaOH at room temperature for 24h., this treatment was used to cleave ester bonds. Following hydrolysis, the extract was neutralized with 1N. HCl and the sample re-extracted in chloroform (section 2.6.1.2). The aqueous fraction remaining after the first hydrolysis was then subjected to a second hydrolysis with an equal volume of 1N. H₂SO₄ at 90°C for 2h., this treatment was used to cleave glycosidic bonds (the treatment will also cleave esters and for that reason this hydrolysis was performed second) (Harborne, 1984). Released phenolics were then extracted into chloroform as before and the 3 extracts were then analysed using 1D-TLC (section 2.6.2.1) and RITA (section 2.6.2.2). The results are presented in Table 3.3.1b.

Alkaline hydrolysis released radiolabelled chloroform-soluble compounds (Table 3.3.1b.) and a large increase in the amount of radiolabel recovered in the chloroform fraction was measured (5632 dpm). Further hydrolysis with acid did not release any more radiolabelled chloroform -soluble compounds. The increased release of radiolabelled compounds following alkaline hydrolysis was investigated further using 1D-TLC (section 2.6.2.1.) and RITA (section 2.6.2.2.). The results presented in Fig.3.3.1d. show that there was no release of radiolabelled phenylpropanoids or capsaicinoids upon alkaline hydrolysis since all intermediates (other than vanillylamine) move from the origin upon 1D-TLC.

Possibly the increased radiolabel in the chloroform fraction was due to cleavage of an ester bond of a vanillylamine conjugate. Further analysis would be required to clarify the point. Capsaicinoids formed do not appear to form esters or glycosides, subsequent analysis therefore concentrates on recovery of the free amide in the chloroform fraction.

Table 3.3.1b.

Distribution of radiolabel in the aqueous and chloroform fractions following alkaline hydrolysis (B), acid hydrolysis (C), and no hydrolysis (A).

treatment	total radiolabel in the fraction (dpm)	
	aqueous fraction (dpm.($\times 10^{-6}$))	chloroform fraction (dpm.($\times 10^{-3}$))
A	2.1	3.8
B	1.8	5.6
C	1.7	1.1

Fig. 3.3.1d.

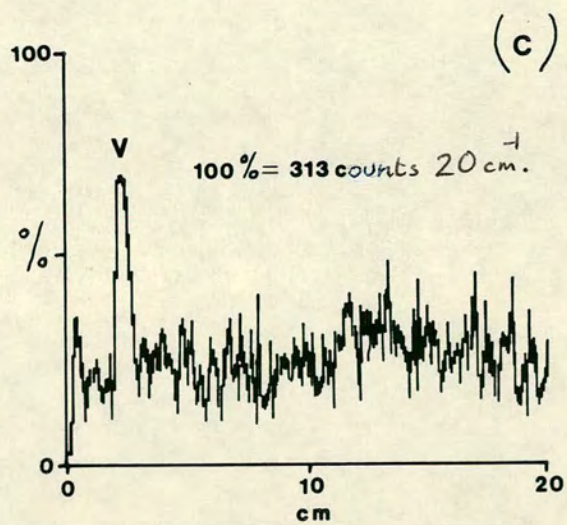
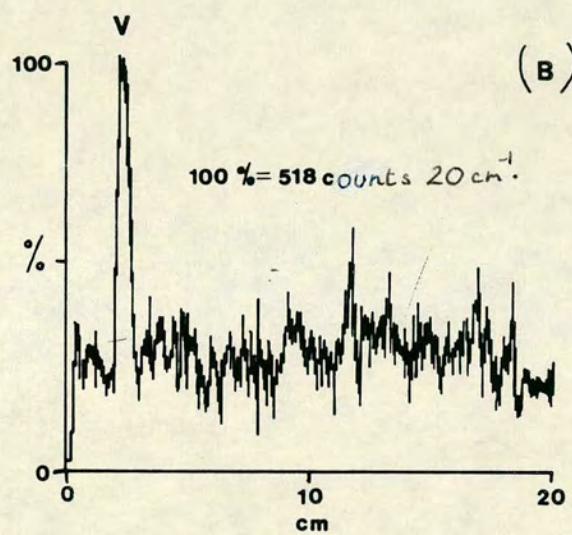
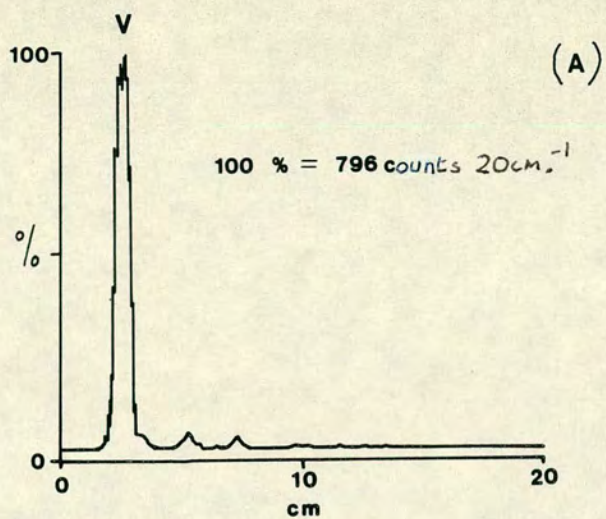
RITA traces of samples separated by 1D-TLC following hydrolysis to test for the presence of esters and/or glycosides.

(A) = no hydrolysis treatment.

(B) = alkaline hydrolysis.

(C) = acid hydrolysis.

where V = vanillylamine



From the data presented in section 3.3.1. it can be seen that a significant back reaction involving the deamination of vanillylamine to vanillin takes place in the assay tube. High levels of capsaicinoids are present in the extract and it is likely that they are causing negative feedback inhibition of capsaicin synthase and thus limiting the rate of the forward reaction. This experiment was designed to attempt to remove end-product inhibition by removal of capsaicinoids.

3.3.2. Testing the efficiency of removal of capsaicinoids from a crude cell-free extract of fruits of *C.frutescens* using dialysis or PD-10 Sephadex chromatography.

For the reasons outlined above, it was decided that a method for the efficient removal of capsaicinoids from the extract was required. This experiment compares dialysis and PD-10 chromatography as possible methods for the efficient removal of capsaicinoids.

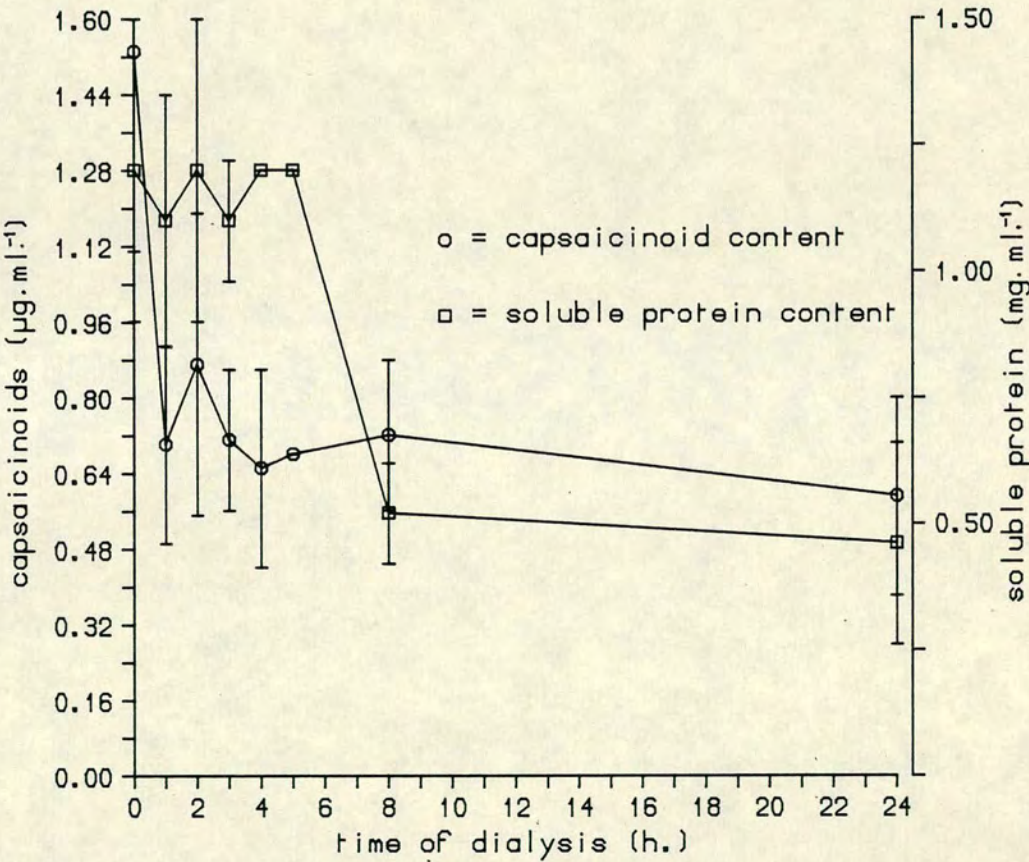
A crude cell-free extract of fruits of *C.frutescens* was prepared as for experiment 3.3.1. and treated in one of three ways: The extract aliquots (A), (B) and (C) were subjected to a) no treatment (control), b) dialysis and c) PD-10 Sephadex chromatography respectively.

Dialysis: Dialysis tubing (Medicell International Ltd., UK. 2-18,32 inches) was boiled for 10 min. (M^cPhie, 1977) in a large volume of 2% sodium bicarbonate and 1mM EDTA, after which it was rinsed thoroughly in distilled water. The tubing was then boiled in distilled water for 10 min. and allowed to cool before a final wash inside and out with distilled water. Dialysis was carried out at 4°C in 20mM potassium phosphate buffer (pH 6.8) containing 3mM EDTA and 5mM β -mercaptoethanol. Separate dialysis vessels were set up covering a time course from 1-24h. The buffer was changed 3 times in the first hour and then dialysis was continued for 24h. during which, samples were removed at 1, 2, 5, 10 and 24h. intervals. The extract was removed from the tubing and an aliquot was taken for soluble protein determination (section 2.5.2.3). Each extract was then partitioned with chloroform and extracted as described in section 2.6.1.2. Capsaicinoid content was determined by HPLC analysis (section 2.6.3.).

Fig. 3.3.2.

The efficiency of removal of capsaicinoids from a crude cell-free extract of fruits of *C.frutescens*. The soluble protein content of the extract is also shown.

Fig. 3.3.2.



PD-10 Sephadex chromatography: Pre-packed PD-10 Sephadex columns (Pharmacia) were equilibrated as described in section 2.5.1.2. Removal of capsaicinoids from the extract was achieved by applying 2.5ml. of the extract to the top of the column and eluting the protein as described in section 2.5.1.2. Soluble protein content was determined as before and the eluate was extracted into chloroform. HPLC analysis was used to determine capsaicinoid content.

The results presented in Fig. 3.3.2. show the removal of capsaicinoids from the crude enzyme extract over 24h. The capsaicinoid content decreased significantly during the first hour of dialysis after which no significant decrease occurred up to 24h. when the capsaicinoid content was approximately 40% of the initial value. The soluble protein content remained constant over the first 5h. of dialysis treatment but dropped to less than 50% of the initial value after 5h. This was probably due to the dilution effect whereby water is taken up into the tubing as a result of osmotic differences between the external bathing medium and the extract.

The results presented in Table 3.3.2. show the efficiencies of removal of capsaicinoids from the extract using PD-10 Sephadex chromatography. A comparison of the two figures in the first column shows that capsaicinoids were removed efficiently from the extract using this method. Following PD-10 chromatography, the capsaicin content was less than 2% of the initial value. In column 2, it can be seen that there was a decrease in the soluble protein content of the extract following PD-10 chromatography (*ca.* 43%), however, the decrease in protein content was not significantly different from the decrease observed following dialysis (*ca.* 50%).

Capsaicinoid removal from the crude cell-free extract is effected more efficiently with PD-10 Sephadex chromatography than with dialysis. The protein concentration of the extract is reduced in both cases and choice of a suitable method for the removal of capsaicinoids must take into account the time required to 'purify' the extract. For all of these reasons it was decided that PD-10 Sephadex chromatography represents the most efficient method for the removal of capsaicinoids from crude cell-free extracts of chilli pepper fruits.

Table 3.3.2.

The efficiency of removal of capsaicinoids from a crude cell-free extract of fruits of *C.frutescens*.

extract	capsaicinoid content ($\mu\text{g.ml}^{-1}$)	soluble protein content (mg.ml^{-1})
crude extract	2.36	1.20
following PD-10 chromatography	0.037	0.69

From the data presented in section 3.3.2. it can be seen that PD-10 chromatography is effective for the removal of capsaicinoids from crude cell-free extracts obtained from fruits of *C.frutescens*. Before inclusion of this step in the extraction procedure, it was important to confirm that enzyme activity would not be lost following PD-10 chromatography.

3.3.3. Measurement of the incorporation of radiolabel from [^3H]vanillylamine into capsaicinoids and vanillin before and after PD-10 Sephadex chromatography.

This experiment was designed to test whether PD-10 Sephadex chromatography affects the incorporation of [^3H]vanillylamine into capsaicinoids and vanillin. In addition, detergent solubilization of the preparation was attempted to see if increased incorporation into capsaicinoids could be achieved. Detergent solubilization of some enzyme preparations has been shown to increase the levels of activity of certain membrane-associated enzymes.

Fruits of a variety of ages were harvested to ensure that capsaicin synthase activity would be present in the extract. The fruits were weighed and extracted as described in section 3.3.1. The supernatant obtained from the 1000xg spin was divided into 3 aliquots, the first of which was used as a control cell-free extract. The second was subjected to PD-10 Sephadex chromatography (section 2.5.1.) and the eluted protein fractions were combined and used as a source of capsaicin synthase. The third fraction was treated with 1% Triton X-100 to solubilize any membrane proteins. The proteins were then separated from the detergent by low speed centrifugation on a sucrose cushion (Bordier, 1981) and

subjected to PD-10 chromatography as described before. All three extracts were assayed for capsaicin synthase activity as described in section 2.5.2.2 [system 2] and final analysis of radiolabel incorporation into capsaicin was achieved by HPLC analysis (section 2.6.3.) and liquid scintillation counting (2.6.4.)

The results of the experiment are presented in Tables 3.3.3a. and 3.3.3b. In Table 3.3.3a. the distribution of radiolabel between the chloroform and aqueous fractions can be seen. In all reaction assays the amount of label found in the chloroform fraction increased compared with the control. This was consistent with the idea that a chloroform-soluble compound had been formed during the assay. The percentage recovery of radiolabel was in excess of 89% in all cases.

From Table 3.3.3b. it can be seen that in the crude cell-free extract no label was found in the capsaicinoids. This was probably due to negative feedback on capsaicin synthase. Following PD-10 Sephadex chromatography there was a small amount of incorporation into capsaicinoids. Incorporation was not increased by this detergent treatment. The incorporation of label into vanillin was high (approximately 2% of the total label supplied) in all treatments and did not alter following PD-10 Sephadex chromatography. It can be seen in the first column of Table 3.3.3b. that the soluble protein content of the extract was lower following PD-10 Sephadex chromatography and Triton X-100 treatment did not increase the soluble protein content of the extract. The activities of capsaicin synthase and vanillylamine deaminase were not lost following PD-10 Sephadex chromatography. The technique can therefore be included as part of the capsaicin synthase extraction procedure.

Table 3.3.3a.

The distribution of radiolabel in selected fractions as a percentage of the total label supplied* following a 2h. incubation of a fruit extract with [³H]vanillylamine.

extract	distribution of label (as % of total supplied)			
	control or reaction	aqueous fraction	chloroform fraction	% recovery
crude extract	control reaction	90	4	94
		82	11	93
PD-10 extract	control reaction	90	5	95
		71	23	94
detergent solubilization	control reaction	91	4	95
		70	20	90

* = amount of label supplied was the same for each assay tube.

Table 3.3.3b.

The rates of conversion of [³H]vanillylamine into vanillin and into capsaicinoids in fruit extracts of *C.frutescens*.

extract	conversion rate		
	soluble protein (µg.ml ⁻¹)	capsaicinoids (dpm. h. ⁻¹ mg. ⁻¹)	vanillin (dpm. h. ⁻¹ mg. ⁻¹ (x10 ⁻⁵))
crude extract	1.6	0.0	2.0
PD-10 extract	1.2	8,036	1.9
detergent solubilization	1.1	7,382	2.0

The data presented in section 3.3.3. show that there is some incorporation of radiolabel into capsaicinoids following PD-10 Sephadex chromatography of the enzyme extract. The results show that vanillylamine deaminase also functions very effectively after PD-10 chromatography however, the presence of this reaction poses problems when attempting to optimize conditions for the forward reaction.

3.3.4. Centrifugal fractionation of a crude cell-free extract of fruits of *C.frutescens* in order to attempt the separation of capsaicin synthase and vanillylamine deaminase activities.

The present experiment used low-speed centrifugation to fractionate the crude extract and attempted to separate the vanillylamine deaminase activity from that of capsaicin synthase. Some evidence has suggested that capsaicin synthase may be associated with the tonoplast (Fujiwake *et al.*, 1982). If so, this should make it possible to separate the two activities since many plant oxidative deaminases are soluble proteins (Mann, 1955).

Fruits of selected ages were used in this experiment chosen at ages where there was a maximum chance of detecting capsaicin synthase activity (20–25 d.). The fruits were weighed and extracted in buffer as described in section 2.5.1.2. Once filtered through Miracloth, 10ml. of the filtrate was retained as crude cell-free extract (A). All operations were carried out at 4°C. The remainder of the extract was centrifuged at 1000xg. for 10 min. to pellet cell wall material, PVP-bound polyphenolics, nuclei and whole cells. Following centrifugation, the pellet was retained (B) and the supernatant spun again at 10,000xg. for 20 min. This pelleted the mitochondria, golgi, some endoplasmic reticulum and plasma membrane. The pellet was retained (C) and the supernatant subjected to a final spin at 24,000xg. This pelleted the plasma membrane, E.R, tonoplast and other membrane fragments (D). The supernatant was retained as fraction E. All pellets were resuspended separately in extraction buffer and subjected to PD-10 Sephadex chromatography to remove endogenous capsaicinoids. The protein fractions eluted from the column in each case were combined and assayed for protein content (section 2.5.2.3) and capsaicin synthase activity (section 2.5.2.2 [system 2.]). The reaction mixtures were extracted into chloroform (section 2.6.1.2) and analysed using HPLC (section 2.6.3.) and liquid scintillation counting (section 2.6.4.).

The results presented in Table 3.3.4a. show the distribution of radiolabel in the aqueous and chloroform fractions for each of the different assays. The highest conversion into chloroform-soluble compounds occurred in fraction E (supernatant) where incorporation was 14% of the total label supplied. Fraction D showed 5% incorporation of the label supplied. The percentage recovery in each case was >87%. The soluble protein content of each fraction was measured and can be seen in the final column of Table 3.3.4a.

The rate of incorporation of radiolabel into capsaicinoids and vanillin is shown in Table 3.3.4b. There was no incorporation into capsaicinoids in fractions A and B and only very low levels of label were incorporated in fraction C. In fraction D (the pellet obtained from the 24,000xg. spin), there was a high level of incorporation into capsaicinoids (*ca.* 4% of the total radiolabel supplied). In fraction E (soluble supernatant) trace levels of label were found in capsaicinoids but incorporation into vanillin was very high (1.6×10^5 dpm. $\text{h}^{-1}\text{mg}^{-1}$). Incorporation into vanillin in all other fractions was also high.

The results suggest that the deaminase is possibly a soluble enzyme and for that reason it was found distributed throughout the fractions. The data support the idea that capsaicin synthase activity may be associated with a membrane (possibly the tonoplast) but further investigation using tonoplast preparations would be required to confirm this point. Medium speed centrifugation could be used as a useful preliminary purification step for the capsaicin synthase enzyme.

Table 3.3.4a

Distribution of label as a percentage of the total radiolabel supplied in the chloroform-soluble and the aqueous fractions. Protein content in the fruit enzymic extracts is shown in the final column.

assay	% of the total radiolabel supplied			
	chloroform soluble fraction	aqueous fraction	% recovery	protein content (mg.ml ⁻¹)
A CON	3	94	97	2.2
A REAC	6	92	98	2.2
B CON	4	95	99	1.84
B REAC	4	93	97	1.84
C CON	4	91	95	2.64
C REAC	6	86	92	2.64
D CON	5	83	88	2.2
D REAC	10	89	99	2.2
E CON	4	88	92	2.0
E REAC	18	78	96	2.0

Table 3.3.4b.

Table showing the rate of incorporation of radiolabel into capsaicinoids and vanillin in the different enzyme fractions.

assay	rate of incorporation of radiolabel	
	capsaicinoid (dpm.h. ⁻¹ mg. ⁻¹)	vanillin (dpm.h. ⁻¹ mg. ⁻¹ (x10 ⁻⁵))
A	0	3.0
B	0	3.6
C	365	2.0
D	96,382	4.0
E	3,268	1.6

where: CON = control assay tube
REAC = reaction assay tube

A = crude cell-free extract
B = pellet from 1,000xg. spin
C = pellet from 10,000xg. spin
D = pellet from 24,000xg. spin
E = supernatant

3.3.5. Determination of the pH optimum for capsaicin synthase extracted from fruits of *C.frutescens*.

In general, enzymes are only active over a limited range of pH and in most cases a definite pH optimum is observed. This experiment was designed to obtain a pH profile for capsaicin synthase and from the profile, to determine the pH optimum for capsaicin synthase activity. In addition, the experiment tests for irreversible destruction of the enzyme over a selected pH range.

Fruits of *C.frutescens* (of a wide range of ages) were harvested, weighed and extracted (section 2.5.1.2). An aliquot of the eluate obtained after PD-10 Sephadex chromatography was assayed for soluble protein content (section 2.5.2.3), the remainder was used as a source of capsaicin synthase activity. Buffered solutions (0.2M Tris-HCl, 3mM EDTA and 5mM β -mercaptoethanol) were prepared over the pH range 6.5 to 9.5. All substrates were made up at the relevant buffered pH (section 2.5.2.2) and radioactive assays were run (3 replicates per treatment) at each pH (section 2.5.2.2:[system 2]). The reaction mixtures were extracted with chloroform (section 2.6.1.2) and analysis of incorporation into capsaicinoids was achieved using HPLC (section 2.6.3.) and liquid scintillation counting (section 2.6.4.).

From the results presented in Fig. 3.3.5a. it can be seen that the pH optimum for capsaicin synthase activity falls between pH 7.0 and 8.0 with a maximum rate of incorporation of radiolabel at pH 7.5 (on this scale) of 1.5×10^3 dpm $\text{h}^{-1}\text{mg}^{-1}$. The pH profile for vanillylamine deaminase appeared much broader with some activity detected over the entire pH range tested. There was a very high maximum rate of incorporation into vanillin (9.5×10^3 dpm. $\text{h}^{-1}\text{mg}^{-1}$) which occurred at pH 8.5. The experiment was repeated over a narrow pH range to attempt to pin-point more exactly where the pH optimum for capsaicin synthase might be.

The results presented in Fig. 3.3.5b. show the data obtained from the second pH profile. The maximum rate of incorporation into capsaicinoids occurred at pH 7.4 (*ca.* 2.0×10^3 dpm. $\text{h}^{-1}\text{mg}^{-1}$). Either side of pH 7.4 there was a significant decline in capsaicin synthase activity. The activity of vanillylamine deaminase increased with increasing pH but was not at a maximum at the same pH as capsaicin synthase.

Fig. 3.3.5a.

The incorporation of radiolabel from [^3H]vanillylamine into vanillin and capsaicinoids in an *in vitro* assay over a wide range of pH treatments.

Fig. 3.3.5b.

The incorporation of radiolabel from [^3H]vanillylamine into vanillin and capsaicinoids in an *in vitro* assay over a narrow range of pH treatments.

Fig. 3.3.5a.

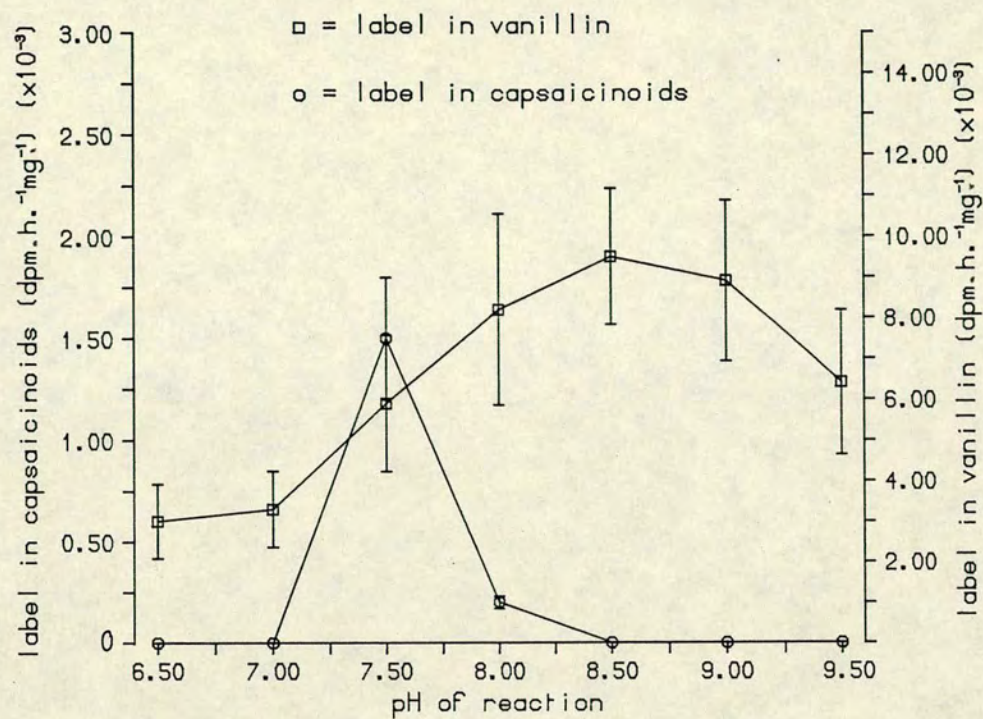
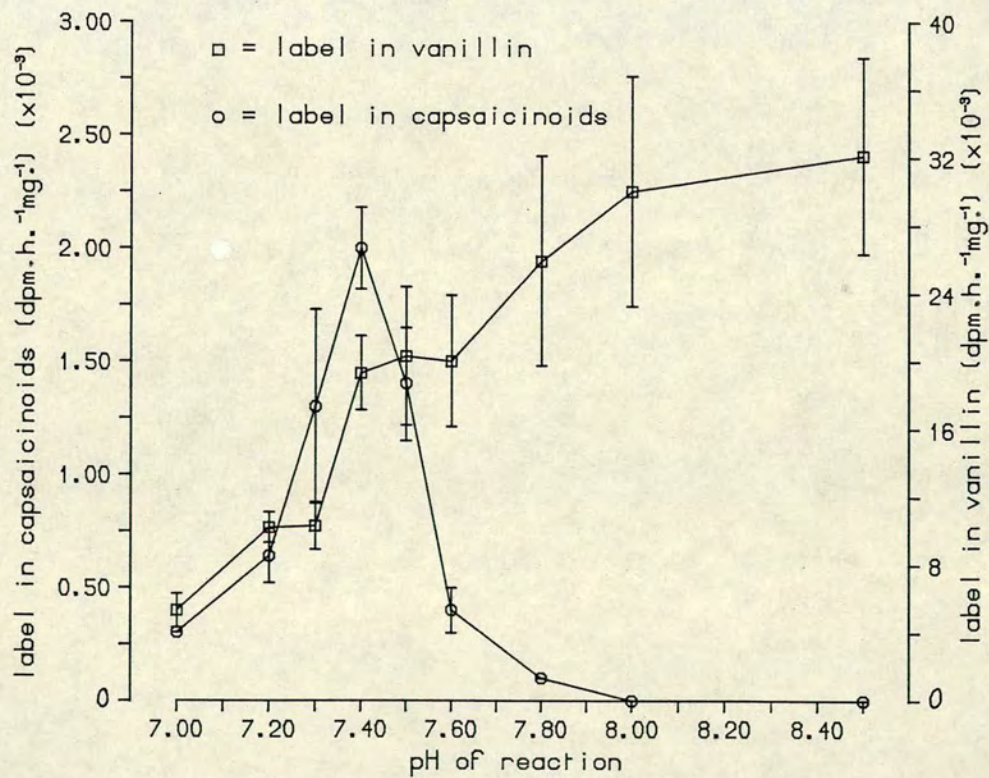


Fig. 3.3.5b.



The irreversible denaturation of capsaicin synthase was tested by exposing aliquots of the enzyme extract for 5 min. to a wide range of pH values, the activity was then tested at pH 7.4 (the putative pH optimum for capsaicin synthase). The universal buffer mixture was chosen for this wide pH range rather than a combination of two buffer systems so that observed differences would be due to pH effects rather than changes in buffer regimes. The assays and subsequent analyses were completed as before.

From the results presented in Fig. 3.3.5c. it can be seen that the pH optimum for capsaicin synthase was *ca.* 7.5 (curve (A)). No capsaicin synthase activity was observed at the extreme low and high pH treatments. In curve (B) the destructive nature of acidic pH on the activity of capsaicin synthase can be seen. Activity was not recovered upon restoration of the pH to 7.4 from low pH treatments. At higher pH values (7.0–9.0), there was SUBSTANTIAL recovery of capsaicin synthase activity upon restoration of the pH from alkaline conditions to pH 7.4. The pH optimum for capsaicin synthase extracted from fruits of *C.frutescens* was determined to be *ca.* 7.4 therefore in subsequent experiments the enzyme was assayed at this pH. The pH optimum for vanillylamine deaminase was found to be fairly broad (somewhere between pH 7.5 and 9.5).

So far, all experiments in section 3.3. have concentrated on maximizing capsaicin synthase activity and although several improvements have been made to the original extraction and assay procedures, there is still a substantial back reaction converting [³H]vanillylamine into vanillin.

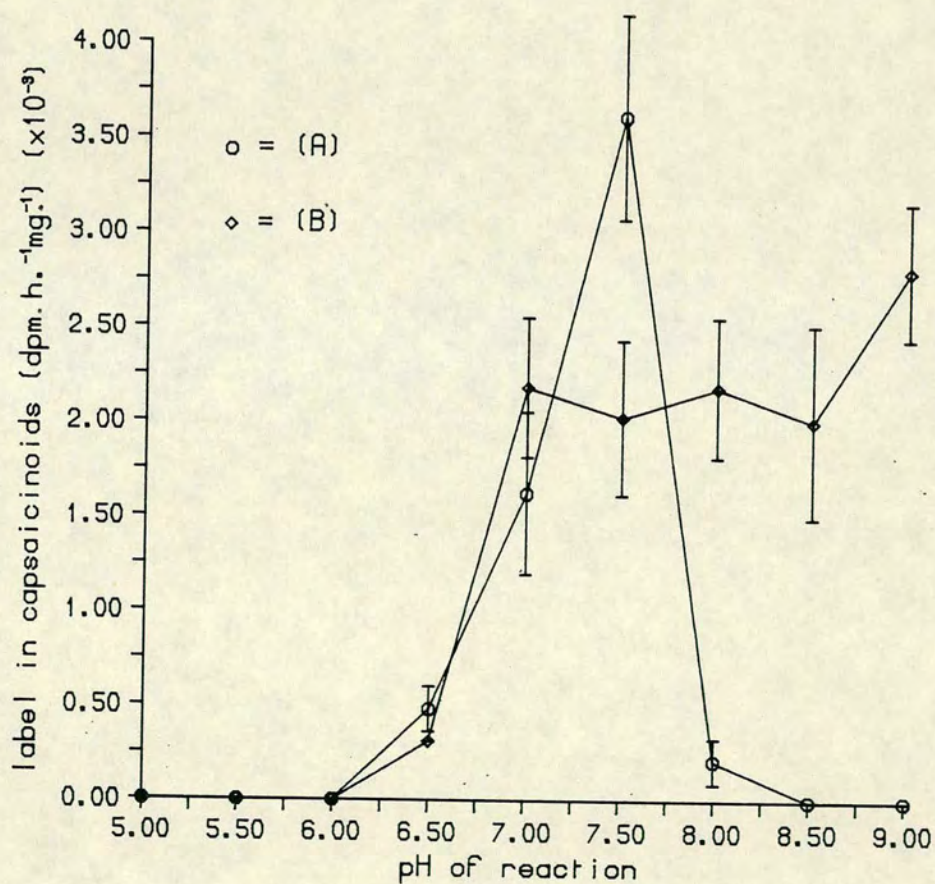
Fig. 3.3.5c.

Testing for irreversible denaturation of capsaicin synthase after various pH treatments:

(A) = enzyme incubated and assayed at pH shown.

(B) = enzyme subjected to pH shown for 5min. and then assayed at pH 7.4.

Fig. 3.3.5c.



3.3.6. Attempts to reduce the vanillylamine deaminase reaction.

The experiments in this section were all designed to reduce the vanillylamine deaminase reaction in an attempt to maximize conversion of radiolabel into capsaicinoids.

3.3.6.1. The use of selected treatments to provide reducing conditions for the *in vitro* assay of capsaicin synthase.

In this experiment, selected treatments were chosen to provide reducing conditions in the assays. Vanillylamine deaminase requires oxidative conditions for activity so the assay treatments attempt to find the most effective way of decreasing vanillylamine deaminase activity.

Fruits of selected ages were harvested, weighed and extracted according to the method described in section 2.5.1.2. The protein fractions eluted from the PD-10 column were combined and used as a source of capsaicin synthase. Four separate assays were run (3 replicates of each) and for each, the reaction buffer was prepared as follows:

(A) = 0.2M Tris-HCl buffer, pH 7.4.

(B) = (A) + 10mM β -mercaptoethanol.

(C) = (A) + 5mM ascorbate.

(D) = (A) + 1mM NADPH₂.

The assays were run according to section 2.5.2.2 [system 2] and the reaction mixtures extracted with chloroform (section 2.6.1.2). The samples were analysed by HPLC (section 2.6.3.) and liquid scintillation counting (section 2.6.4.).

The results presented in Table 3.3.6.1. show the rates of incorporation of radiolabel from vanillylamine into capsaicinoids and vanillin for all of the reducing treatments used. It can be seen that only one treatment resulted in capsaicin synthase activity, the presence of β -mercaptoethanol resulted in high levels of radiolabel in capsaicinoids (*ca.* 2% of the label supplied). Assays where buffer without β -mercaptoethanol was used did not show capsaicin synthase activity and neither did the presence of ascorbate or NADPH₂ increase capsaicin synthase activity. The incorporation into vanillin was *ca.* an order of

magnitude greater than the incorporation into capsaicinoids in treatment (B). Most vanillylamine deaminase activity was measured in assay (A) as might be expected since the conditions were most oxidative in this system. Incorporation into vanillin was lowest when β -mercaptoethanol was used as the reducing agent (6% of the total supplied) and was *ca.* 3 times greater when ascorbate or NADPH₂ were used in the assay (16% and 18% respectively of the total radiolabel supplied).

Table 3.3.6.7.

The rates of incorporation of radiolabel from vanillylamine into capsaicinoids and vanillin under different reducing conditions.

Treatment	Rate of incorporation of label (dpm.h. ⁻¹ mg. ⁻¹ (x10 ⁻⁴))	
	Capsaicinoids	Vanillin
(A)	0.0 ±0.0	59 ± 5.4
(B)	3.8 ±0.41	11 ±1.2
(C)	0.0 ±0.0	29 ±1.9
(D)	0.0 ±0.0	32 ±3.3

where: (A) = 0.2M.Tris-HCl, pH 7.4

(B) = (A) + 10mM β -mercaptoethanol

(C) = (A) + 5mM ascorbate

(D) = (A) + 1mM NADPH₂

each result is the mean of three replicates ± standard error.

Since β -mercaptoethanol was found to be required for capsaicin synthase activity *in vitro*, subsequent experiments utilize this reducing agent in both the extraction and assay buffers.

3.3.6.2. Limiting the vanillylamine deaminase reaction by supplying vanillin and glutamine in the assay mixture.

This experiment was designed to try and limit the rate of the vanillylamine deaminase reaction by supplying vanillin and an NH_2 donor (glutamine) in an attempt to force the equilibrium in the direction of capsaicin synthesis.

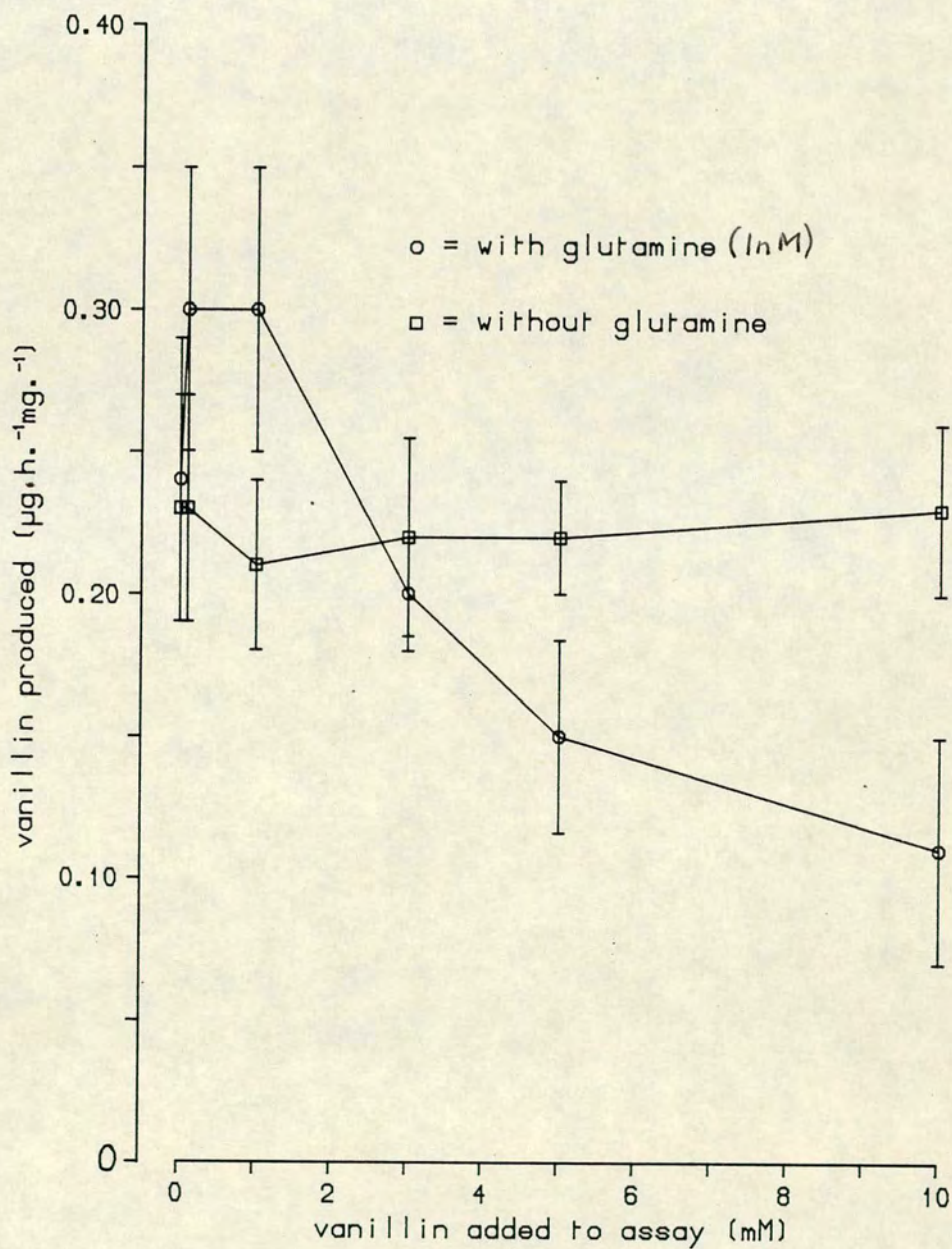
Fruits (20–25 d.) of *C. frutescens* were harvested, weighed and extracted (section 2.5.1.2.). The protein fractions eluted from the PD-10 column were combined and used as the source of capsaicin synthase. Non-radioactive assays were run as described in section 2.5.2.2. [system 1] with the addition of vanillin and glutamine^(1mM) or vanillin only. The experiment was replicated three times. The reaction mixtures were extracted into chloroform (section 2.6.1.2) and the samples were analysed using HPLC (section 2.6.3.). Vanillin production was measured using the calibration curves presented in section 2.6.3.4.

The data presented in Fig.3.3.6.2. show the synthesis of vanillin in the presence of vanillin with and without glutamine. Over the concentration range of vanillin used (0–10 mM final concentration) there was no significant decrease in the rate of the vanillylamine deaminase reaction in the absence of glutamine. However, the rate was slightly reduced (from $0.3 \mu\text{g} \cdot \text{h}^{-1} \cdot \text{mg}^{-1}$ to $0.1 \mu\text{g} \cdot \text{h}^{-1} \cdot \text{mg}^{-1}$) with increasing vanillin concentration in the presence of glutamine. Vanillin does not appear to feed-back regulate vanillylamine deaminase, only in the presence of glutamine (at fairly high concentrations) does vanillin have an effect on the activity. The data are not sufficiently conclusive for the technique to be used in subsequent assays to limit the vanillylamine deaminase reaction.

Fig. 3.3.6.2.

Vanillin production in the presence of increasing concentrations of vanillin and in the presence/absence of glutamine.

Fig. 3.3.6. (2).



3.3.6.3. Use of methylene blue and semicarbazide in an attempt to inhibit vanillylamine deaminase activity *in vitro*.

In an attempt to reduce vanillylamine deaminase activity, two known inhibitors were used in this experiment. Methylene blue is a potent inhibitor of amine oxidase [E.C. 1.4.3.4.] but not other oxidases (Philpot and Cantoni, 1941). An important feature of amine oxidase is that it is not inhibited by carbonyl reagents eg. semicarbazide, however, there is a group of other enzymes which catalyse the oxidative deamination of amines [E.C. 1.4.3.6.] and these are inhibited very strongly by such reagents. This group of oxidases can thus be distinguished from the classical amine oxidase because of inhibitor specificity. The present experiment was designed to reduce the activity of vanillylamine deaminase using specific inhibitors with the aim of using the technique subsequently (*in vitro*) to increase incorporation into capsaicinoids.

Fruits (20–25 d.) of *C.frutescens* were harvested, weighed and extracted (section 2.5.1.2). The protein fractions eluted from the column were combined and used as a source of capsaicin synthase. Non-radioactive and radioactive assays were run as described in section 2.5.2.2 [system 1] and [system 2]. The inhibitors were added to the assays at the following final concentrations:

methylene blue = 2mM (Philpot and Cantoni, 1941)

semicarbazide = 3mM (Hayward and Large, 1981)

The experiment was replicated 3 times. The reaction mixtures were extracted into chloroform (section 2.6.1.2) and the non-radioactive samples were analysed using HPLC (section 2.6.3.). The radioactive samples were analysed using HPLC in conjunction with liquid scintillation counting (section 2.6.4.).

The results presented in Table 3.3.6a. show the rate of vanillin formation (column 1) and the incorporation of radiolabel into vanillin (column 2) in the presence and absence of the inhibitors. The results show that semicarbazide was an effective inhibitor of vanillylamine deaminase (*ca.* 50% reduction in activity in presence of semicarbazide). Methylene blue did not affect the rate of the vanillylamine deaminase reaction. In Table 3.3.6b. the incorporation of radiolabel into capsaicinoids can be seen in the presence and absence of inhibitors. The data show that there was no significant increase in the rate of

the capsaicin synthase reaction in the presence of either inhibitor. Although semicarbazide reduced the rate of the "back" reaction by 50%, the "forward" reaction was not increased, this would suggest that availability of vanillylamine in the assay system as a substrate for capsaicin synthase is not a limiting factor.

Table 3.3.6a.

The effect of semicarbazide and methylene blue on the vanillylamine deaminase reaction *in vitro*.

inhibitor	vanillin produced ($\mu\text{g} \cdot \text{h}^{-1} \text{mg}^{-1}$)	incorporation into vanillin ($\text{dpm} \cdot \text{h}^{-1} \text{mg}^{-1} (\times 10^{-5})$)
no inhibitor	0.38 ± 0.05	5.0 ± 0.09
+ semicarbazide	0.21 ± 0.07	2.5 ± 0.09
+ methylene blue	0.40 ± 0.14	6.1 ± 0.07

Table 3.3.6b.

Effect of semicarbazide and methylene blue on the incorporation of radiolabel from [^3H]vanillylamine into capsaicinoids in an *in vitro* assay.

inhibitor	incorporation into capsaicinoids ($\text{dpm} \cdot \text{h}^{-1} \text{mg}^{-1} (\times 10^{-4})$)
no inhibitor	2.4 ± 0.2
+ semicarbazide	2.8 ± 0.5
+ methylene blue	2.7 ± 0.4

The results presented in section 3.3.6. have provided some useful information on the vanillylamine deaminase reaction. As a result of this series of experiments, β -mercaptoethanol was used in all subsequent *in vitro* analyses. Vanillin, glutamine, semicarbazide and methylene blue were not used in subsequent assays. Experiments in section 3.3. have concentrated on the development of an assay procedure for capsaicin synthase utilizing radioactive vanillylamine. Although further optimization of the extraction and assay procedures would undoubtedly increase the sensitivity of the capsaicin synthase assay, it was decided that the assay was sufficiently optimal to make comparative measurements of capsaicin synthase activity *in vitro*.

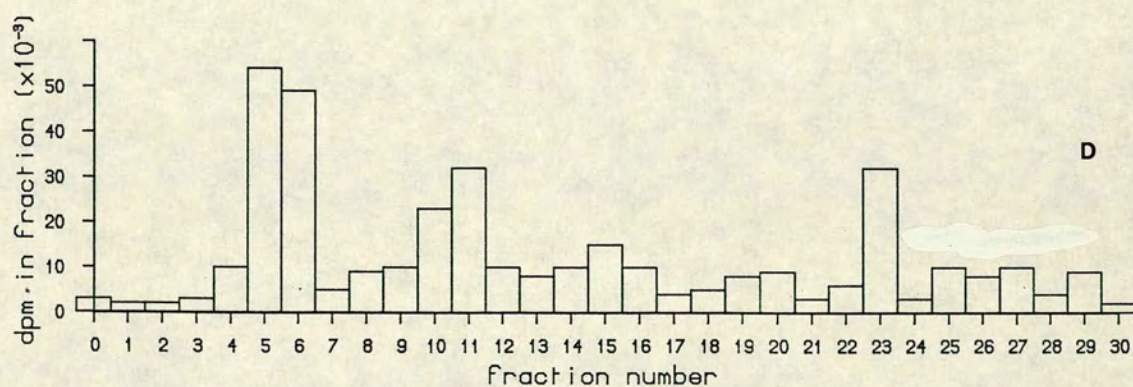
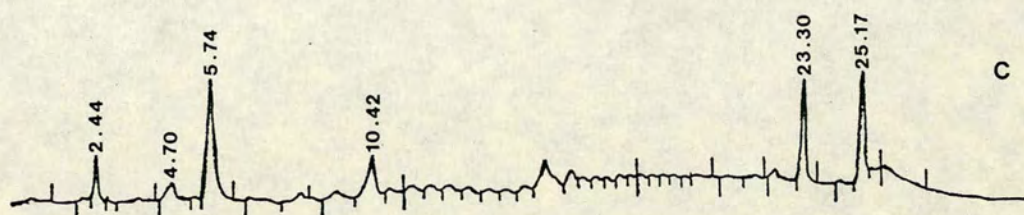
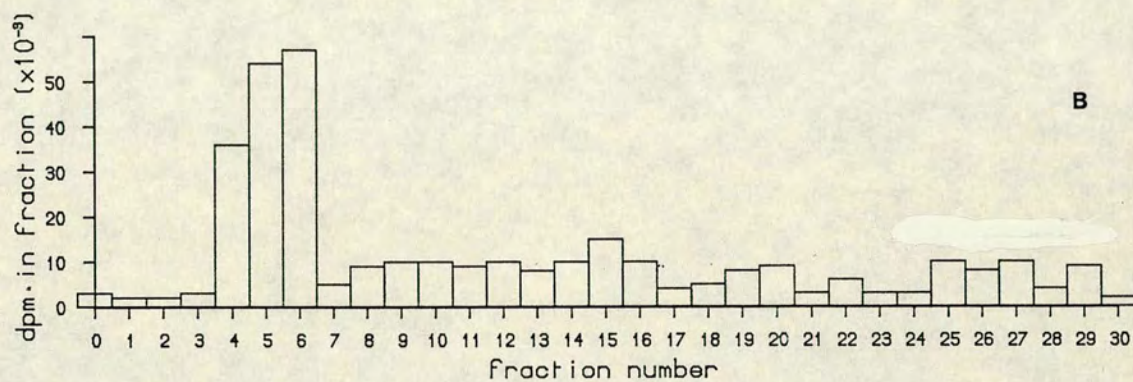
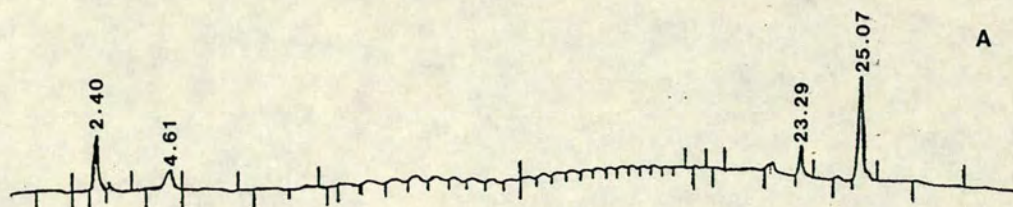
3.3.7. Measurement of capsaicin synthase activity using: a) a radioactive assay and b) a non-radioactive assay.

This experiment was designed to test if the assay developed so far was good enough to allow measurement of quantities of capsaicinoids produced during the assay to be detected using HPLC rather than liquid scintillation counting of incorporated label. A radioactive assay was carried out on the same extract for comparison.

Fruits (20–25 d.) were harvested, weighed and extracted as described in section 2.5.1.2. Following PD-10 Sephadex chromatography, the protein fractions eluted from the column were combined and used as a source of capsaicin synthase. Assays were run using the radioactive assay procedure (section 2.5.2.2 [system 2]) and the non-radioactive assay procedure (section 2.5.2.2 [system 1]). The reaction mixtures were extracted with chloroform (section 2.6.2.) and the radioactive samples were analysed using HPLC (section 2.6.3.) and liquid scintillation counting (section 2.6.4.). The non-radioactive samples were analysed by HPLC and the quantities of capsaicinoids produced were calculated from the calibration curves (section 2.6.3.4).

Fig. 3.3.7.

HPLC traces ((A) and (C)) of control and reaction samples respectively shown with radioactivity profiles ((B) and (D)) obtained after counting fractions eluted from the HPLC column. Trace (A) corresponds with (B) and Trace (C) corresponds with (D).



The results presented in Fig. 3.3.7. demonstrate that quantities of capsaicinoids detectable by HPLC were produced during the assay procedure. The HPLC traces A and C show the non-radioactive control and reaction assays respectively. The most noticeable differences between these two are the peaks at 5.74 min. (vanillylamine), 10.42 min. (vanillin) and 23.30 min. (dihydrocapsaicin) which are either absent or greatly reduced in the control systems. Capsaicin synthase activity therefore appears to be sufficient to produce significant quantities of capsaicinoids under the assay conditions used. The data presented in Table 3.3.7. show the production of vanillin and capsaicinoids in the assay system. No capsaicinoids were produced in the assay tube containing crude cell-free extract and a significant deaminase reaction resulted in the formation of vanillin ($0.48 \mu\text{g} \cdot \text{h}^{-1} \text{mg}^{-1}$). Following PD-10 Sephadex chromatography however, capsaicinoids were produced at measurable levels ($0.32 \mu\text{g} \cdot \text{h}^{-1} \text{mg}^{-1}$) and the amount of vanillin produced was lower (compared with the crude extract). The radioactive assay results support these data. Traces B and D show the incorporation of radiolabel from [^3H]vanillylamine into capsaicinoids and vanillin in the control and reaction assays respectively. In trace B no radiolabel is found in any compound other than vanillylamine. In trace D however, there is incorporation of radiolabel into vanillin (10.42 min.) and dihydrocapsaicin (23.30 min.).

The data presented in 3.3.7. show that capsaicin synthase can be assayed using the radioactive or non-radioactive assay procedures with a reasonable degree of reliability.

Results from Part 3.3. have shown the development of an assay procedure for capsaicin synthase. Conditions found to be most suitable for the assay of the enzyme are:

1. Extract subjected to PD-10 Sephadex chromatography to remove capsaicinoids.
2. Centrifugal fractionation to separate vanillylamine deaminase from capsaicin synthase.
3. Assay at pH 7.4.
4. Reducing conditions maintained throughout extraction and assay procedures with β -mercaptoethanol.

It was recognized that further optimization of the assay and purification of the enzyme would improve the sensitivity and convenience of the procedure, however, for the purposes of subsequent comparative experiments, the assay procedure was considered sufficiently optimal.

Table 3.3.7.

The production of capsaicinoids and vanillin in an *in vitro* system at concentrations detectable by HPLC.

	amount of product formed ($\mu\text{g. h.}^{-1} \text{ mg.}^{-1}$)	
	capsaicinoids	vanillin
PD-10 extract	0.32 ± 0.19	0.21 ± 0.14
crude extract	0.00 ± 0.00	0.48 ± 0.21

Data presented in section 3.3. have provided a crude optimal assay procedure for capsaicin synthase. In section 3.3.7. the assay was shown to be sufficiently sensitive to detect levels of capsaicinoids produced *in vitro* using HPLC.

Part 3.4. Partial characterization of the capsaicin synthase reaction(s).

The present section provides a partial characterization of the reaction(s) catalysed by capsaicin synthase, essential for the understanding of the likely mechanism of action of the enzyme.

3.4.1. Determination of the cofactor and substrate requirements of capsaicin synthase.

This experiment was designed to confirm the cofactor and substrate requirements of capsaicin synthase. The radioactive assay was used because it is a more sensitive system.

Fruits of *C. frutescens* (20–25 d. following fruit initiation) were harvested, weighed and extracted as described in section 2.5.1.2. The extract was subjected to centrifugal fractionation (section 3.3.4.) and the pellet obtained from the 24,000xg. spin was resuspended in buffer and subjected to PD-10 Sephadex chromatography (section 2.5.1.2). The assays were run as described in section 2.5.2.2 [system 2] but in each case (except the control) one component of the reaction mixture was omitted. Where a cofactor or substrate was left out, an appropriate volume of buffer was added to compensate for the reduced volume. The reaction mixtures were extracted in chloroform (section 2.6.1.2) and analysed using HPLC (section 2.6.3.) and liquid scintillation counting (section 2.6.4.).

The results of the experiment are presented in Table 3.4.1. From the data it can be seen that vanillylamine deaminase has no requirement for ATP, Mg^{2+} , CoASH or 8-methyl-6 *nonanoic* acid. The rate of incorporation into vanillin was similar in each case (*ca.* 3×10^5 dpm·h⁻¹ mg⁻¹) which represented about 2% of the total radiolabel supplied. Incorporation into capsaicinoids occurred at a rate of *ca.* 1.9×10^4 dpm·h⁻¹ mg⁻¹ in the complete assay system. An absolute requirement for ATP and CoASH was shown by the absence of incorporation where either of these cofactors was left out. Some activity was measured in the absence of $MgCl_2$, possibly because there was sufficient endogenous Mg^{2+} present in the extract for the reaction to proceed. When 8-methyl-6 *nonanoic* acid was omitted from the assay, some incorporation into capsaicinoids occurred, this was due to incorporation into capsaicin rather than dihydrocapsaicin. In this situation the [³H] vanillylamine condensed with endogenous 8-methyl-6-nonenoic acid (rather than 8-methyl-6 *nonanoic* acid)

to form capsaicin. These data show that CoASH and ATP are required for capsaicin synthase activity.

Table 3.4.1.

The incorporation of radiolabel from [³H]vanillylamine into capsaicinoids and vanillin in assays where putative essential cofactors and substrates had been omitted.

incorporation of radiolabel (dpm.h. ⁻¹ mg. ⁻¹)				
assay system	capsaicinoids		vanillin	
complete	19,389	± 1,879	196,382	± 13,642
-ATP	0	± 0	361,320	± 21,329
-MgCl ₂	1,021	± 436	296,382	± 19,835
-CoASH	0	± 0	304,829	± 23,647
-ICA	964	± 439	286,031	± 17,054

where: ICA = 8-methyl acid

The data presented in section 3.4.1. suggest that capsaicin synthase has an absolute requirement for ATP and CoASH. This supports the idea that capsaicin synthase consists of at least two components; an acyl-activating enzyme which activates the acyl group to a CoA-activated moiety and an acyl transferase enzyme which transfers the acyl-CoA derivative onto vanillylamine. Both of these enzyme types have been documented in plant systems.

3.4.2. Utilizing selected CoA-activated derivatives to test for the presence of an acyl transferase enzyme in a cell-free extract of fruits of *C.frutescens*.

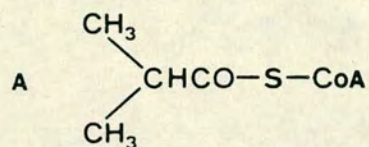
This experiment was designed to attempt to assay one of these putative enzymes (acyl transferase) in a crude cell-free extract of chilli fruits. The following selected CoA- activated derivatives were purchased from Sigma (Fig. 3.4.2a.):

Fruits of *C.frutescens* (20-25 d.) were harvested, weighed and extracted (section 2.5.1.2). The protein fractions obtained following PD-10 Sephadex chromatography were combined and used as a source of the acyl transferase enzyme. Separate radioactive assays were run for each of the 4 CoA-activated derivatives. ^(1MM) In addition, a standard capsaicin synthase assay was run (section 2.5.2.2 [system 2]) using 8-methylnonanoic acid. The assays involving activated derivatives were not supplied with ATP, CoASH or MgCl₂ and the controls in each case were minus CoA-activated derivatives. All other parameters for the assays were as described in section 2.5.2.2 [system 2]. The reaction mixtures were partitioned with chloroform (section 2.6.1.2) to extract both natural and un-natural capsaicinoids formed during the assay procedure. All samples were qualitatively analysed using 1D-TLC (section 2.6.2.1) and RITA (section 2.6.2.2) to identify radioactive peaks in the samples.

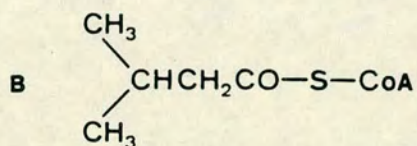
The results presented in Table 3.4.2. show the distribution of radiolabel between the chloroform and aqueous fractions in both the control and reaction samples. In all assays there was an increase in the amount of radiolabel found in the chloroform fractions of the reaction mixture compared with the controls. This supported the idea that a chloroform-soluble compound had been synthesized during the assay. The highest conversion to chloroform- soluble compounds from [³H]vanillylamine occurred when 8-methylnonanoic acid was used as a substrate. High levels of incorporation into chloroform-soluble compounds were also observed when Isovaleryl-CoA was used as a substrate.

Fig. 3.4.2a.

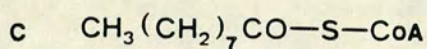
Structures of the four CoA-activated derivatives obtained from Sigma (A,B,C,D) along with the two putative CoA-activated derivatives of 8-methyl-nonanoic acid (E) and 8-methyl-6-nonenic acid (F).



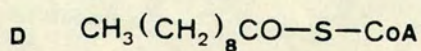
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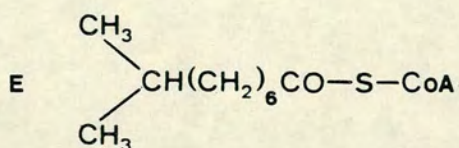
Isovaleryl-CoA (5C)



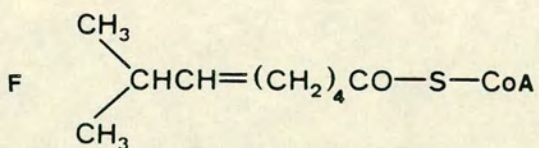
n-Nonanoyl-CoA (9C)



n-Decanoyl-CoA (10C)



8-Methylnonanoyl-CoA (10C)



8-Methylnonenoyl-CoA (10C)

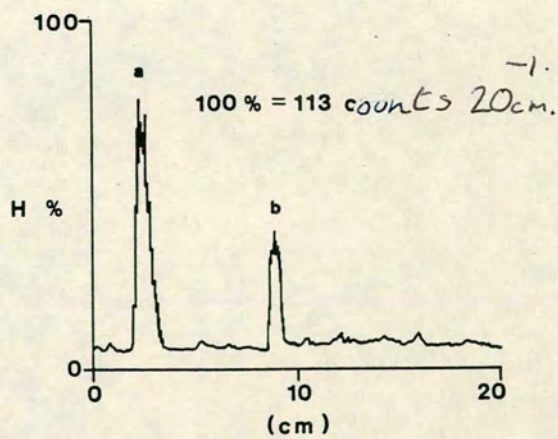
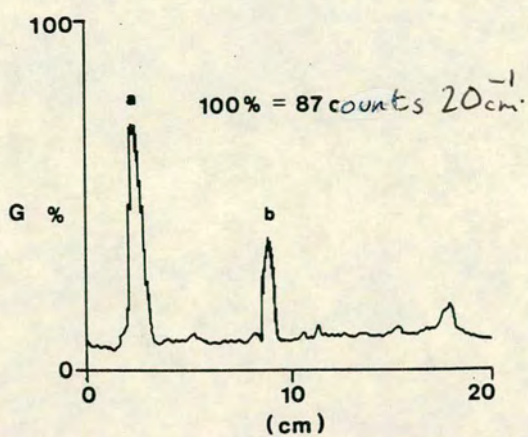
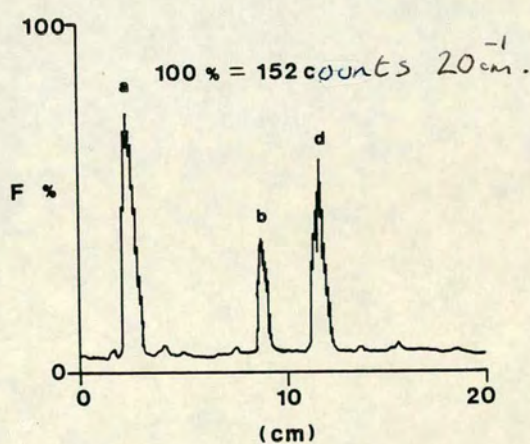
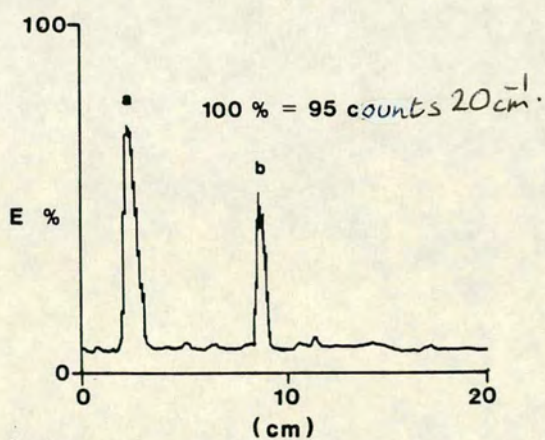
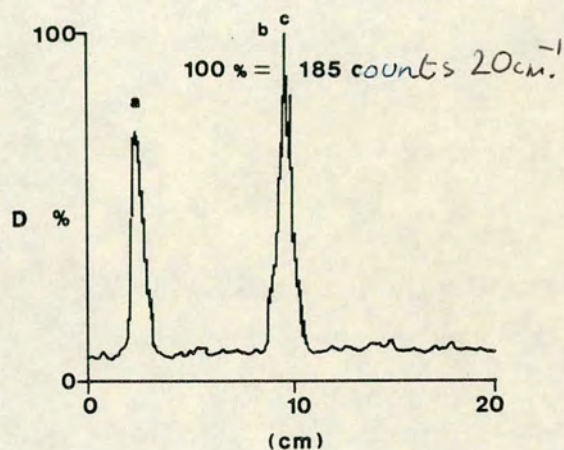
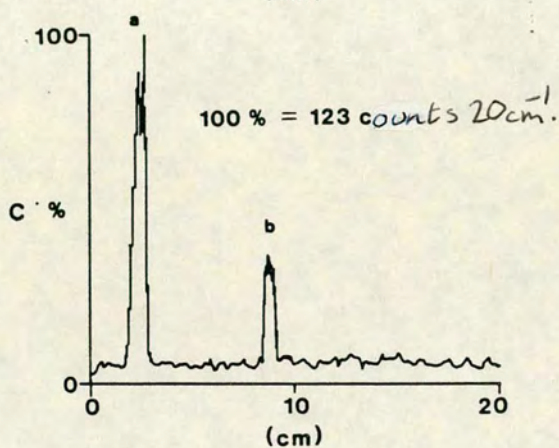
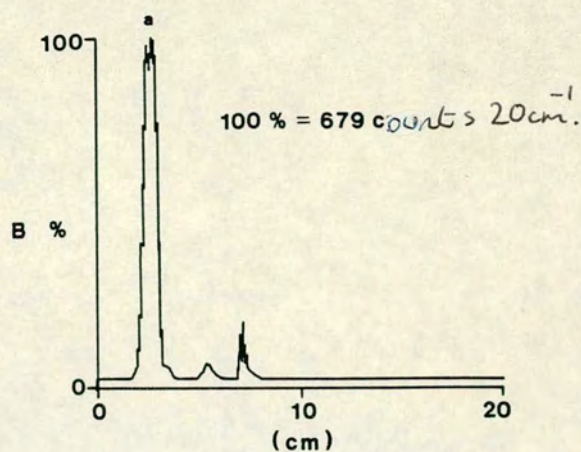
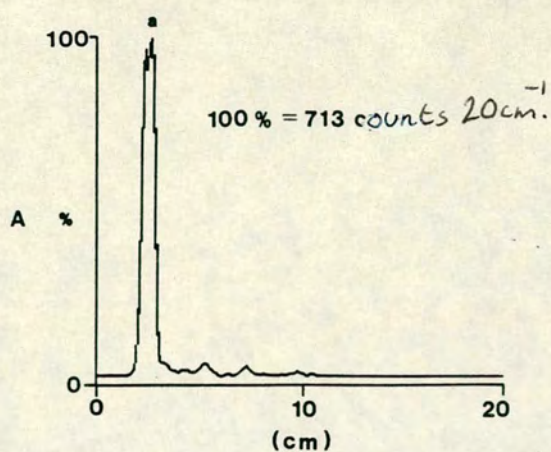
Fig. 3.4.2b.

RITA traces of a number of samples:

- A = control aqueous fraction
- B = reaction aqueous fraction
- C = control chloroform-soluble fraction
- D = reaction chloroform fraction with
8-methyl *nonanoic* - acid as substrate
- E = reaction chloroform fraction with
Isobutyryl-CoA as substrate
- F = reaction chloroform fraction with
Isovaleryl-CoA as substrate
- G = reaction chloroform fraction with
n-Nonanoyl-CoA as substrate
- H = reaction chloroform fraction with
n-Decanoyl-CoA as substrate

where:

- a = vanillylamine
- b = vanillin
- c = capsaicinoid
- d = capsaicinoid-type product



In Fig. 3.4.2b. the results of the RITA analysis can be seen. Traces A and B were obtained following 1D-TLC and RITA of control and reaction aqueous fractions. All aqueous fractions in this experiment showed the same pattern of radiolabel distribution, predominantly in vanillylamine. Trace C is representative of the chloroform soluble control samples following incubation of the control system with [^3H]vanillylamine. A large proportion of label remained at the origin in vanillylamine and a small peak of radiolabel was found at a position which coincided with vanillin. This was to be expected since the stock [^3H]vanillylamine slowly converts to vanillin. Traces D to H are of the chloroform-soluble reaction samples. 8-methylnonananoic acid was used as the precursor in trace D and the peak found at 9.5cm represents radiolabelled dihydrocapsaicin. Isobutyryl-CoA was the substrate in trace E and radioactivity was found only in vanillylamine and vanillin as for the control. The result in trace F was obtained using Isovaleryl-CoA as the CoA-activated derivative, here label was found in vanillylamine and vanillin, as for the control. A peak of radioactivity was also observed at ca. 12cm and was most likely an un-natural capsaicinoid-type compound. Traces G and H were obtained using n-Nonanoyl-CoA and n-Decanoyl-CoA respectively, neither of these two substrates appeared to combine with vanillylamine and no radioactive peaks other than vanillylamine and vanillin were detected. Substrates that were effective in combining with [^3H]vanillylamine to form chloroform-soluble labelled compounds were 8-methylnonananoic acid and Isovaleryl-CoA. A comparison of the structures of the acyl-activated derivatives (see Fig. 3.4.2a.) demonstrates the importance of the branch chain on the fatty acid for transfer to vanillylamine.

The data presented in Part 3.4. show that capsaicin synthase has an absolute requirement for ATP and CoASH, MgCl_2 is also required for the reaction to occur. In addition it was shown that an acyl transferase enzyme is present in fruits of *C.frutescens*. The enzyme appears to have a certain degree of specificity for CoA-activated iso-type fatty acids.

Table 3.4.2.

Distribution of radiolabel in chloroform and aqueous fractions for control and reaction samples in assays utilizing selected CoA-activated derivatives as substrates.

substrate	assay	distribution of radiolabel (% of total supplied)	
		chloroform fraction	aqueous fraction
no acyl group	CON	4	90
	REAC	10	80
8-methyl nonanoic acid	CON	5	89
	REAC	16	81
Isovaleryl-CoA (C5)	CON	3	91
	REAC	13	82
Isobutyryl-CoA (C4)	CON	4	89
	REAC	9	83
n-Decanoyl-CoA (C10)	CON	4	91
	REAC	10	80
n-Nonanoyl-CoA (C9)	CON	4	82
	REAC	9	81

where:

CON = control assay
REAC = reaction assay

The aim of the experiments described in section 3.3. and 3.4. was to develop a sensitive and reliable assay for capsaicin synthase. To some extent this was achieved and a partial characterization of the reaction system has indicated the likely mechanism of action for the enzyme complex. The assay was considered sufficiently sensitive to be used in comparative studies on fruits of *C.frutescens*. It was decided to test whether or not capsaicin accumulated at the same time as the development of capsaicin synthase activity or if enzyme activity was present in the fruit some time before capsaicin accumulation.

Part 3.5. To measure the activity of capsaicin synthase activity throughout fruit development and attempt to correlate it with the onset of capsaicin production *in vivo*.

The aim of the present experiment was to determine a) when capsaicin synthase activity could be detected in the fruit (using the *in vitro* assay), and b) whether capsaicin accumulated at the same time as capsaicin synthase activity appeared.

Fruits of exactly the same age were selected. Fruit (A) left intact and attached was injected with [³H]vanillylamine (section 2.4.2.) (ca. 1 μCi. per fruit) and incubated with the radiolabel for 6h; fruit (B) was harvested, weighed and extracted (section 2.5.1.2.) to determine capsaicin synthase activity. The protein fractions eluted from the PD-10 Sephadex column were combined and used as a source of capsaicin synthase activity. Enzyme activity was assayed as described in section 2.5.2.2. and the reaction mixtures extracted into chloroform (section 2.6.1.2.). Following the 6h. *in vivo* incubation with radiolabel, fruit (A) was sliced longitudinally and washed twice with buffer to remove exogenous [³H]vanillylamine. The fruit was then dissected into placenta and pericarp and each extracted separately in methanol (section 2.6.1.1.). All fractions were analysed using HPLC (section 2.6.3.) and liquid scintillation counting (section 2.6.4.)

The data presented in Table 3.5. show that the uptake of radiolabelled vanillylamine into the fruit was fairly constant over the experiment. In Fig.3.5a. the results of the *in vivo* experiment are shown. Fruits younger than 7 d. were not used since they were too small to be injected with a sufficient volume of radiolabel. It can be seen that there was no incorporation of label into capsaicinoids before day 17 (Fig. 3.5a.). Between day 17 and 18 however, there

was a large increase in the level of incorporation which reached a maximum of (ca. 4×10^5 dpm, g.fr.wt.⁻¹) in the placenta 19d. following fruit initiation. This level remained constant over a period of 5-6 days and possibly longer since measurements were not taken of fruits over 25 d. The pericarp did not accumulate radiolabel to the same extent although there was a significant level of incorporation into capsaicinoids in the pericarp over the period 18-21 d. (ca. 0.8×10^5 dpm, g.fr.wt.⁻¹). Although label was shown to be taken up by the cells (see table 3.5.) throughout the time course, incorporation into capsaicinoids was not detected prior to day 17.

The data presented in Fig. 3.5b. show the rate of incorporation of radiolabel into capsaicinoids *in vitro* throughout the time course of fruit development. No label was incorporated between day 7-9 and fruits younger than 7 d. were not used since they were too small to produce a reasonable enzyme preparation. By day 10, there was significant incorporation into capsaicinoids detected (ca. 2×10^5 dpm, h.⁻¹mg.⁻¹). This value had risen to a maximum rate of incorporation by ca. day 12 (3×10^5 dpm, h.⁻¹mg.⁻¹) and remained at this level over the range 12-25 d. old fruits.

The results of the *in vitro* assays show that capsaicin synthase activity is present in the fruits before capsaicin synthesis and accumulation occur. The results show that although capsaicin synthase activity was detected as early as day 10 after fruit initiation, no incorporation into capsaicinoids occurs until day 18 *in vivo*.

NB In a separate experiment (results not shown), the assay was found to be linear over ca. 3 hours.

Fig. 3.5a.

Incorporation of radiolabel into capsaicinoids *in vivo* following injection of fruits of various ages with [^3H]vanillylamine.

Fig. 3.5b.

Incorporation of radiolabel into capsaicinoids *in vitro* following assay of fruit extracts for capsaicin synthase activity.

Fig. 3.5a.

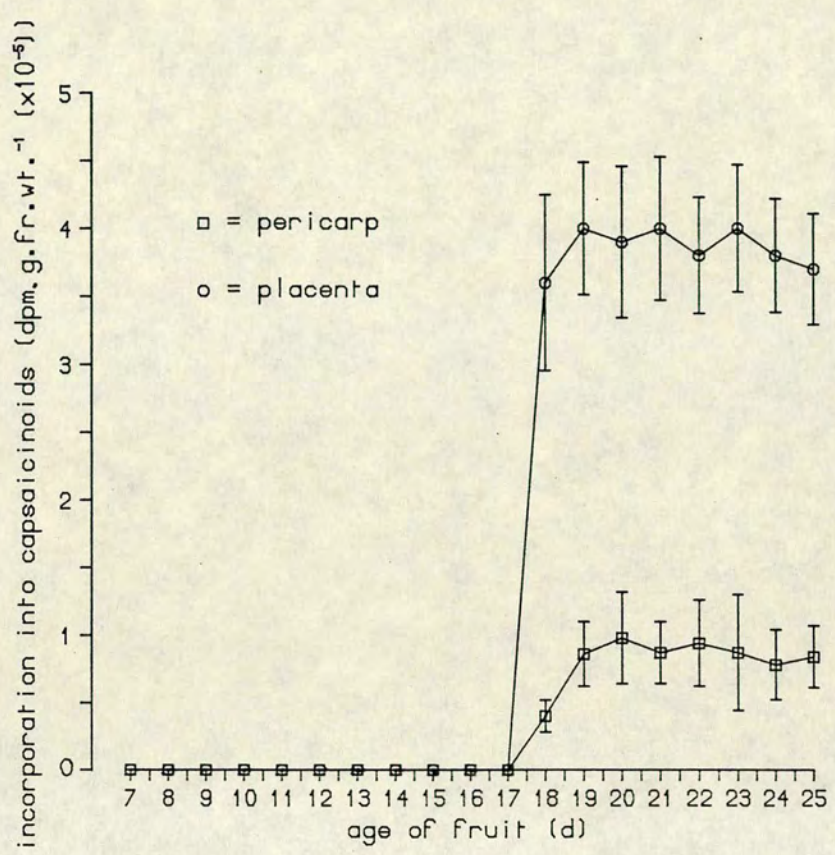


Fig. 3.5b.

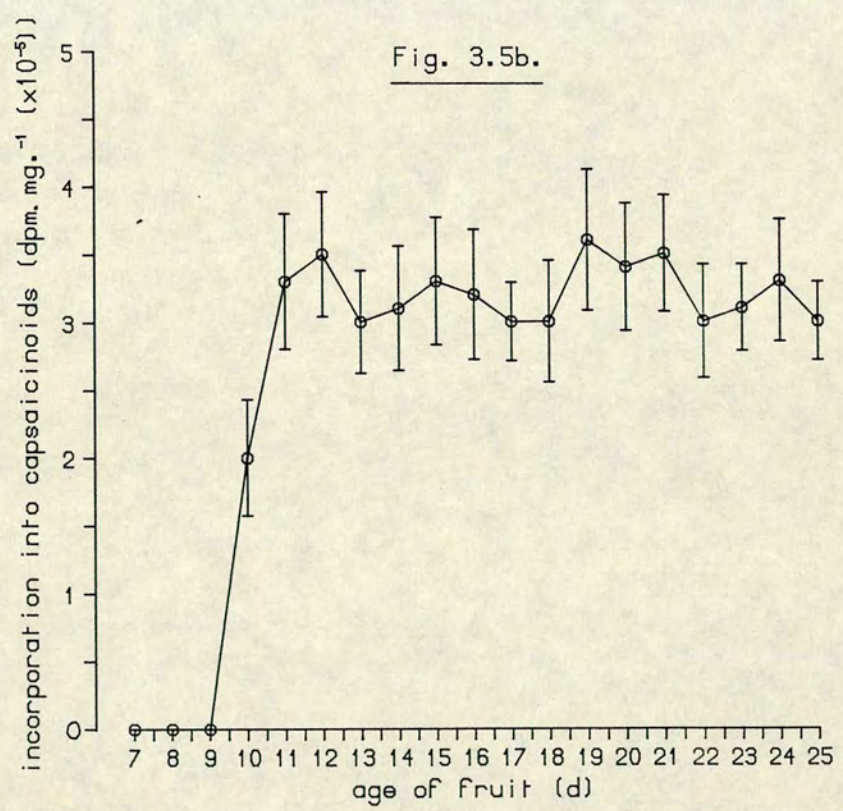


Table 3.5.

The distribution of radiolabel following injection of fruits of different ages with [^3H]vanillylamine.

Age (d)	label in washings (dpm.($\times 10^{-6}$))	label in fruit dpm.($\times 10^{-5}$))	uptake as % of total supplied
7	2.09 \pm 0.36	4.54 \pm 0.52	20.5
8	2.04 \pm 0.42	4.01 \pm 0.49	18.1
9	1.75 \pm 0.38	4.63 \pm 0.52	20.8
10	0.93 \pm 0.23	5.10 \pm 0.65	23.0
11	2.09 \pm 0.38	4.83 \pm 0.56	21.8
12	2.11 \pm 0.57	4.69 \pm 0.38	21.1
13	2.18 \pm 0.27	4.38 \pm 0.61	19.7
14	2.13 \pm 0.36	4.44 \pm 0.45	20.0
15	3.04 \pm 0.54	4.93 \pm 0.53	22.2
16	1.98 \pm 0.43	5.01 \pm 0.62	22.6
17	2.15 \pm 0.35	4.21 \pm 0.39	19.0
18	2.13 \pm 0.62	4.56 \pm 0.28	20.5
19	2.19 \pm 0.54	4.61 \pm 0.39	20.8
20	1.91 \pm 0.37	4.44 \pm 0.61	20.0
21	3.03 \pm 0.54	4.38 \pm 0.54	19.7
22	1.99 \pm 0.29	4.29 \pm 0.71	19.3
23	1.91 \pm 0.48	5.13 \pm 0.43	23.1
24	1.95 \pm 0.27	3.89 \pm 0.54	17.5
25	2.15 \pm 0.35	4.46 \pm 0.28	20.1

each value is the mean of three replicates \pm standard error.

It has been shown that capsaicin accumulates at a particular stage of fruit development and that the appearance of capsaicin synthase activity precedes the onset of capsaicin synthesis (section 3.5.). This may be coincidental or it may be that the appearance of capsaicin synthase is the "switch" required before cells will produce capsaicin. Capsaicin accumulation in the fruit does not immediately follow the increase in capsaicin synthase activity and it is possible that substrate supply may ultimately limit its accumulation. The provision of such substrates in the fruits could come from the release of metabolites following downturn of growth and fruit ripening.

In many cultured cell systems secondary metabolite accumulation has also been correlated with reduced growth rate of cultures (occurs typically in late growth phase and early stationary phase). It is therefore possible that induction of capsaicin synthase activity in cultured cells could occur at a time in the culture cycle associated with downturn of growth. To test this possibility, the following experiment was carried out.

Part 3.6. Measurement of capsaicin synthase activity, capsaicinoid accumulation and flux through the capsaicin pathway in cultured cells of *C.frutescens* subjected to selected culture regimes.

Cultured cells often contain lower levels of enzyme activity than the corresponding parts of the whole plant and because of this it is difficult to develop the assay of novel enzymes in cultured cells. For this reason the assay of capsaicin synthase was developed in fruits (sections 3.3 and 3.4).

The aim of this experiment was to screen cultured cells of *C.frutescens* which had been subjected to selected treatments for capsaicin synthase activity and to relate enzyme activity to capsaicin accumulation and changes in flux through the pathway.

A total of 32 different culture treatments were tested for their ability to induce capsaicin synthase activity. The culture and physical regimes were manipulated and a range of stress treatments were used:

- Nitrogen, to slow growth of cultures and reduce protein synthesis.
- Sucrose, to slow growth of cultures.
- +Ferulate, possible induction of capsaicin synthase activity. regulation.
- +Elicitor, possible induction of capsaicin synthase activity.

The experimental design was complex and the timing of events is set out in Fig. 3.6a.

Fig. 3.6a.

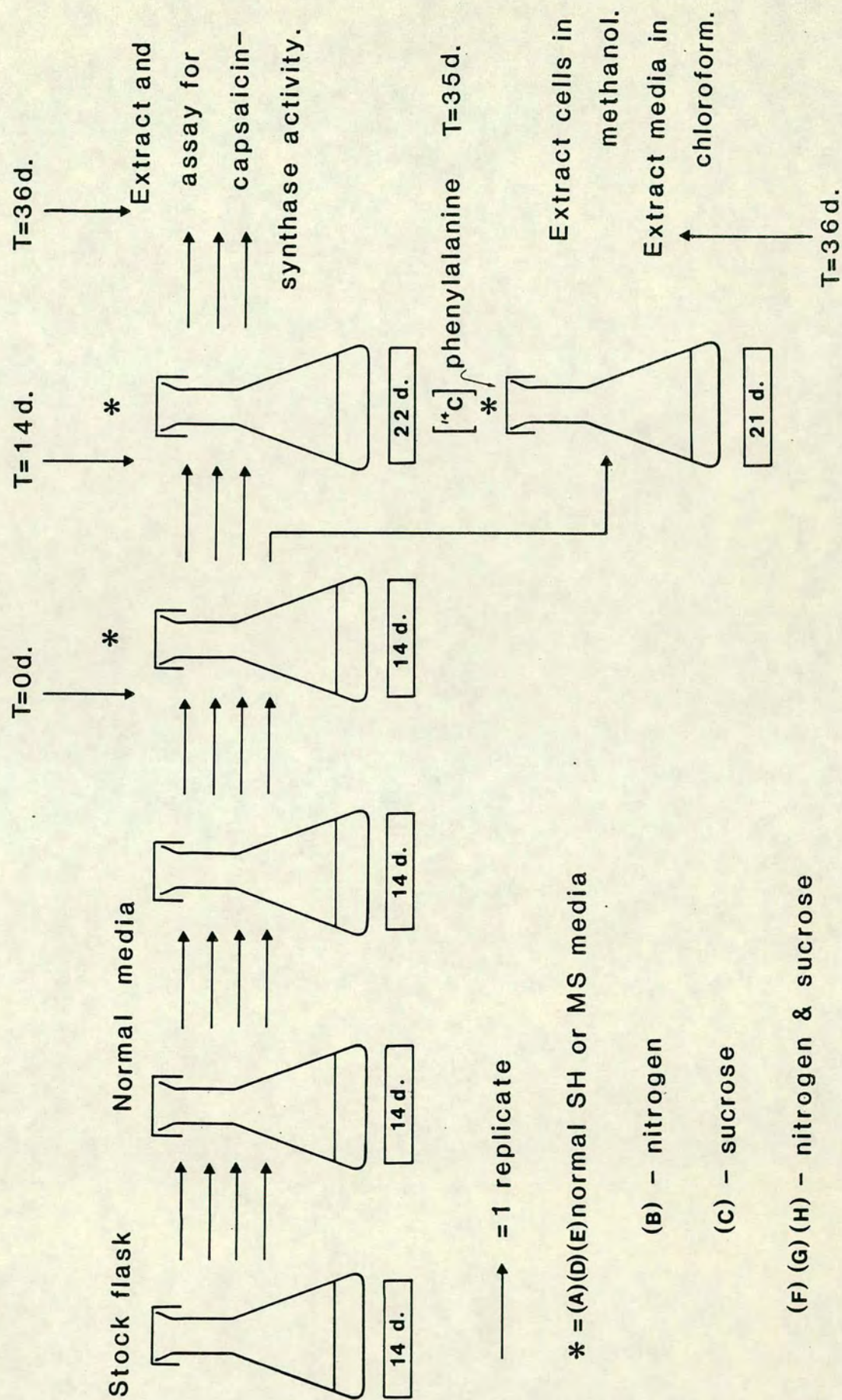
This procedure was carried out for each of the 32 treatments.

For treatments (A), (B), (C) and (F) extraction for capsaicin synthase activity was carried out at T=36d.

For treatments (D), (E), (G) and (H) ferulate/elicitor treatments were added 24h. prior to extraction for capsaicin synthase activity at T=36d.

T=0d. represents the start of the treatment period.

Fig. 3.6a.



Suspended and immobilized cells (section 2.2.2.2) were subcultured (1 block or *ca.* 2.0g. per flask) into SH or MS liquid media (section 2.2.1.1), this resulted in four major treatments.

- (SH)S - suspended cells on SH media
- (MS)S - suspended cells on MS media
- (SH)I - immobilized cells on SH media
- (MS)I - immobilized cells on MS media

Sufficient flasks were inoculated for the entire experiment (treatments were staggered by 24h. to allow sufficient time for sampling). The cells were maintained on the appropriate standard media for two subcultures (a total of 28d.), after which time the stress treatments began (T=0 in Fig.3.6a). The following stress conditions (minor treatments) were applied to the cells:

- (A) = control, subculture onto complete growth medium
- (B) = -nitrogen, subculture onto medium without nitrogen (section 2.2.1.2)
- (C) = -sucrose, subculture onto medium without sucrose (section 2.2.1.2)
- (D) = ferulate feeding, subculture onto complete growth medium
- (E) = elicitation, subculture onto complete growth medium
- (F) = -nitrogen & -sucrose, subculture onto medium without nitrogen and sucrose
- (G) = -nitrogen, -sucrose & ferulate feeding, subculture onto medium without nitrogen and sucrose
- (H) = -nitrogen, -sucrose, ferulate feeding & elicitation, subculture onto medium without nitrogen and sucrose

This resulted in a total of 32 treatments (each of which was replicated 4 times). Each culture was identified with a code:

eg. (SH)S(A)1 = (SH) grown suspended cells on treatment (A), replicate 1.

The cells were grown under stress conditions for 14d., subcultured onto fresh stress media and incubated for a further 21 d. (T=35d.). At T=35d. one

replicate flask for each treatment was injected with [^{14}C] phenylalanine (ca. 1 μCi , per flask) in order to give a measure of flux through the pathway. Ferulate (1mg. per flask) and elicitor ^(spore preparation of *Gliocladium deliquescens*) (15mg per flask) were also added to the appropriate flasks at T=35d. and subsequent harvest took place 24h. later. Ferulate was filter-sterilized and the elicitor was autoclaved prior to addition. All cells were harvested 22 d. into the second treatment subculture (T=36d.) since this was found to be the late growth/early stationary phase of the cultures (appendix A.). Cells which had been supplied with [^{14}C]phenylalanine were extracted in methanol (section 2.6.1.1) and subsequently analysed using 2D-TLC (section 2.6.2.3), autoradiography (section 2.6.2.4) and counted (section 2.6.4.). The media were extracted into chloroform (section 2.6.1.2) and the samples were analysed for capsaicin using HPLC (section 2.6.3.). The non-radioactive cells were extracted for capsaicin synthase activity (section 2.5.1.2) and the supernatant obtained from the 1000xg spin used directly for assay of the enzyme (section 2.5.2.2 [system 2]). Reaction mixtures were extracted into chloroform and the samples were analysed using HPLC (section 2.6.3.) and counted (section 2.6.4.). Soluble protein content was determined as described in section 2.5.2.3.

Table 3.6.

The rates of the capsaicin synthase and vanillylamine deaminase reactions following different culture treatments.

treatment	rate of reaction (dpm.h. ⁻¹ mg. ⁻¹)			
	capsaicin synthase		vanillylamine deaminase	
(SH)S(A)	113.0	± 24.6	nd	
(MS)S(A)	135.3	± 17.6	nd	
(SH)I(A)	nd		1,242.7	± 425.1
(MS)I(A)	nd		836.0	± 266.5
(SH)S(B)	254.0	± 52.6	532.7	± 112.9*
(MS)S(B)	9,350.3	± 907.5*	1,201.0	± 322.0*
(SH)I(B)	nd		nd*	
(MS)I(B)	nd		nd*	
(SH)S(C)	20.3	± 22.4*	444.3	± 101.2*
(MS)S(C)	3,221.7	± 1,074.5*	1.7	± 2.9
(SH)I(C)	776.0	± 194.4	nd*	
(MS)I(C)	453.0	± 366.2	nd*	
(SH)S(D)	nd*		nd	
(MS)S(D)	253.0	± 127.3	43.0	± 55.8
(SH)I(D)	nd		284.0	± 146.6
(MS)I(D)	nd		784.0	± 173.0
(SH)S(E)	425.0	± 110.0*	142.0	± 52.0
(MS)S(E)	2,712.0	± 605.0*	nd	
(SH)I(E)	nd		nd*	
(MS)I(E)	nd		147.0	± 79.0*
(SH)S(F)	nd*		nd	
(MS)S(F)	60.0	± 27.2	nd	
(SH)I(F)	nd		nd*	
(MS)I(F)	nd		nd*	
(SH)S(G)	1,811.7	± 210.4*	700.3	± 149.5*
(MS)S(G)	2,721.0	± 584.9*	1,147.3	± 216.1*
(SH)I(G)	372.7	± 193.2	nd*	
(MS)I(G)	nd		833.7	± 218.0
(SH)S(H)	nd*		nd	
(MS)S(H)	12,509	± 3,711.9*	nd	
(SH)I(H)	nd		199.7	± 141.1
(MS)I(H)	4,600.3	± 828.3*	699.7	± 142.8

nd = not detected

each result is the mean of 3 replicates ± the standard error.

* = significantly different from control mean at the 1% level

The results presented in Table 3.6. show the incorporation of radiolabel from [^3H]vanillylamine into capsaicinoids and vanillin a measure of capsaicin synthase and vanillylamine deaminase activities respectively. All treatment means were compared statistically with the appropriate control mean using the t-test (section 2.7.3.). Values found to be significantly different from the control mean (at the 1% level) are identified in Table 3.6. There was a significant increase in the activity of capsaicin synthase in certain cultures. The highest capsaicin synthase activity was observed in MS grown suspended cells which had been subjected to a combination of all stress treatments ((MS)S(H)), here the rate of incorporation exceeded 1.2×10^4 dpm. h. $^{-1}$ mg. $^{-1}$. High capsaicin synthase activity was also observed using the following treatments; (MS)S(G), (MS)S(E), (MS)S(C), and (MS)S(B). It is interesting to note that the highest levels of capsaicin synthase activity were observed in suspended cells grown on MS media, only one treatment gave a significantly higher level of capsaicin synthase activity in SH grown suspended cells ((SH)S(G)). Cell immobilization did not have a marked stimulatory effect upon the activity of capsaicin synthase. Only two immobilized cell treatments showed significant increases in the levels of capsaicin synthase activity ((SH)I(C) and (MS)I(H)) and of the two, the higher level was found in immobilized cells grown on MS media (4.0×10^3 dpm. h. $^{-1}$ mg. $^{-1}$). HPLC analysis of the chloroform extracts from media and cells showed that no measurable quantities of capsaicinoids had been produced in any of the cultures despite the presence of high capsaicin synthase activity in some cultures.

Vanillylamine deaminase activity was greatly reduced (*cf.* control values) in all immobilized cell treatments. The highest activity of vanillylamine deaminase was detected in control immobilized cells grown on SH media (1.2×10^3 dpm. h. $^{-1}$ mg. $^{-1}$).

It was confirmed using 1D-TLC and RITA (section 2.6.2.1 and 2) that radiolabel in the aqueous fractions remained as unchanged vanillylamine.

Fig. 3.6b.

Autoradiographs showing the patterns of incorporation of label in methanolic extracts of cells after supplying with [^{14}C]phenylalanine for 24h.

(A) = (MS)S(A)

(B) = (MS)S(H)

(C) = (MS)I(A)

(D) = (SH)S(H)

where:

A = phenylalanine

B = unknown, possibly vanillylamine

C = capsaicinoid

D = vanillin

E = cinnamate

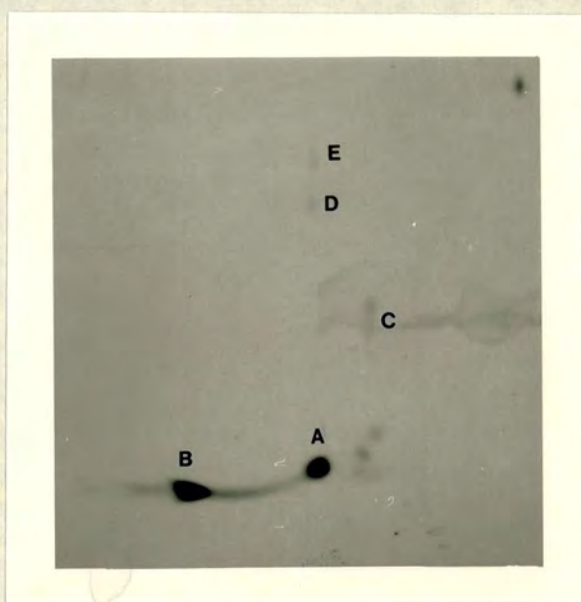
Lettered compounds were identified as described in section 2.6.2.3.

All other labelled compounds were not identified.

(A)

(B)

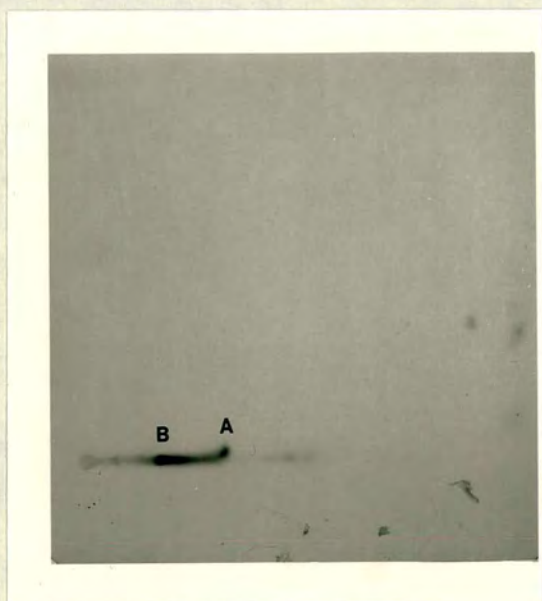
1st. Solvent.



2nd. Solvent.

(C)

(D)



The autoradiographs presented in Fig. 3.6b. show some of the important features of the pattern of incorporation of [^{14}C]phenylalanine when cells of *C.frutescens* are subjected to various culture stress treatments.

One feature usually observed when cells of *C.frutescens* are incubated with [^{14}C]phenylalanine is that immobilized cells tend to incorporate label into more compounds outside the phenylpropanoid pathway than do suspended cells. Dramatic changes in the flux of carbon through the pathway seem to occur when cells are immobilized. This difference can be seen to some extent in Figs. 3.6b.(A) and (C) which show the distribution of radiolabel in suspended and immobilized cells respectively, following stress treatment and incubation with [^{14}C]phenylalanine. Radiolabel can be seen in phenylalanine in both autoradiographs but Fig. 3.6b. (C) has a greater number of unknown compounds labelled when compared with Fig. 3.6b.(A). As can be seen in Table 3.6. in general, MS grown cells tend to have higher capsaicin synthase activity than do SH grown cells. A comparison of Figs. 3.6b (B) and (D) reinforces this point. The cells grown on SH media and subjected to all stress treatments showed no detectable level of incorporation into capsaicin and very little incorporation into any other compound. However, where cells had been grown on MS media and subjected to the same stress treatments incorporation of radiolabel into capsaicinoids, vanillin, cinnamate and a number of other products was observed. This showed that observed differences in capsaicin synthase activity (Table 3.6.) were reflected to some extent by changes in the flux through the capsaicin pathway.

The most obvious correlation which emerged from this experiment is that capsaicin synthase activity appears to be induced when cells are grown on MS media and in particular when there is a reduced nitrogen supply.

Summary

1) Evidence was provided to suggest that PAL and C₄-H do not limit capsaicin production in fruits or cultured cells of *C.frutescens* under the conditions studied. It was apparent that there was no significant increase in the specific activity of these two enzymes under conditions of capsaicin biosynthesis.

2) Using the radiolabelled substrate [³H]vanillylamine, the presence of capsaicin synthase activity was demonstrated *in vivo* in fruits and cultured cells of *C.frutescens*. The [³H]vanillylamine was shown to be taken up and incorporated into capsaicinoids in cells of *C.frutescens* under certain conditions.

3) A method was developed for the extraction and *in vitro* assay of capsaicin synthase activity from fruits of *C.frutescens*. The reaction(s) catalysed by capsaicin synthase was partially characterized and an acyl transferase enzyme with a high degree of specificity for iso-type CoA-activated fatty acids was shown to be active in the enzyme extract.

4) Capsaicin synthase activity was shown to be present in fruits of *C.frutescens* ca. 10 d. prior to capsaicin accumulation. This suggested that substrate limitation may have a role in the regulation of capsaicin biosynthesis *in vivo*.

5) Capsaicin synthase activity was shown to be induced in cultured cells of *C.frutescens* under certain conditions. The highest activity was measured from cells that had been elicited and grown on MS media with reduced nitrogen. The overall pattern of flux through the pathway was demonstrated to be greatly altered as a result of different culture regimes.

Chapter 4

Discussion

Capsaicin (8-methyl-N-vanillyl-6-nonenamide) is the pungent amide accumulated solely in the fruit of *C.frutescens* (Hall *et al.*, 1986). The biosynthesis and accumulation of capsaicin in cell cultures has been studied in this laboratory for a number of years. This work has shown that suspended cells of the chilli pepper accumulate capsaicin in the bathing medium at very low levels (Lindsey and Yeoman, 1984a), although manipulation of the culture conditions by altering the chemical environment eg. nutrient limitation (Yeoman *et al.*, 1980.; Lindsey, 1985), precursor feeding (Lindsey and Yeoman, 1984a,b) elicitation (Holden *et al.*, 1988a,b) and the physical environment eg. cell immobilization (Lindsey *et al.*, 1983.; Lindsey and Yeoman, 1985) all increase the yield of capsaicin. It has also been shown using the radioactive precursors [^{14}C]phenylalanine and [^{14}C]cinnamate that under altered conditions of cell growth, changes occur in the flux through the pathway (Lindsey, 1986). These alterations to the metabolic activity of the cells are reflected in changes in the activities of certain enzymes leading to modifications to the flow of metabolites through the pathway. In order to begin to understand the basis of this biochemical differentiation in cultured cells, it is first necessary to gain a knowledge of how the capsaicin biosynthetic pathway is regulated at the enzyme level. It is reasonable to assume that an understanding of the regulation of the pathway will facilitate manipulation of the balance of metabolism in favour of capsaicin formation and accumulation. For example, it should be possible to identify the enzyme(s) which determine flux through the pathway and increase its (their) activity to increase capsaicin production.

Accordingly selected enzymes in the capsaicin biosynthetic pathway were investigated and the results are now discussed.

Studies on PAL and C,4-H.

The evidence presented in Part 3.1. suggests that PAL and C,4-H are not limiting capsaicin production under the conditions used. It was apparent in these experiments that there was no correlation between the specific activity of PAL (or C,4-H in cultured cells) and the onset of capsaicin synthesis in the fruit or in cultured cells of *C.frutescens*. The results of the studies utilizing AOPP (the selective inhibitor of PAL) are consistent with this finding and point to a limitation effected away from the top of the pathway.

These data are also consistent with previous results of Holden *et al.* (1987a) who showed that the specific activities of PAL and C,4-H were lower in immobilized cells than in suspended cells 12 d. after subculture despite the increased incorporation of radiolabel from [¹⁴C]phenylalanine into capsaicin in immobilized cells. Since the activities of PAL and C,4-H do not always increase before capsaicin accumulation, the extent of capsaicin synthesis may be dependent primarily on the availability of precursors to, and the activity of, enzymes further down the pathway.

Concomitant increases in PAL activity and the levels of phenolic compounds have been demonstrated in many plants and plant tissues (Engelsma, 1967 and Imaseki *et al.*, 1968). However, an increase in the specific activity of PAL does not always correlate with the production of phenylpropanoids (Kleinhofs *et al.*, 1966.; Constabel *et al.*, 1971).; Davies, 1972). It would appear from these examples that PAL is unlikely to be the only site of control of phenylpropanoid metabolism in plants or that changes in the activity of PAL result in the accumulation of phenolics directly in all cases. Indeed Margna, (1977) has suggested that the deaminating capacity of plant tissue is always sufficient to remove phenylalanine surplus to requirements for synthesis of protein and that an increase in accumulation of phenylpropanoid compounds is likely to be due to changes in the supply of phenylalanine to PAL rather than an increase in the catalytic activity.

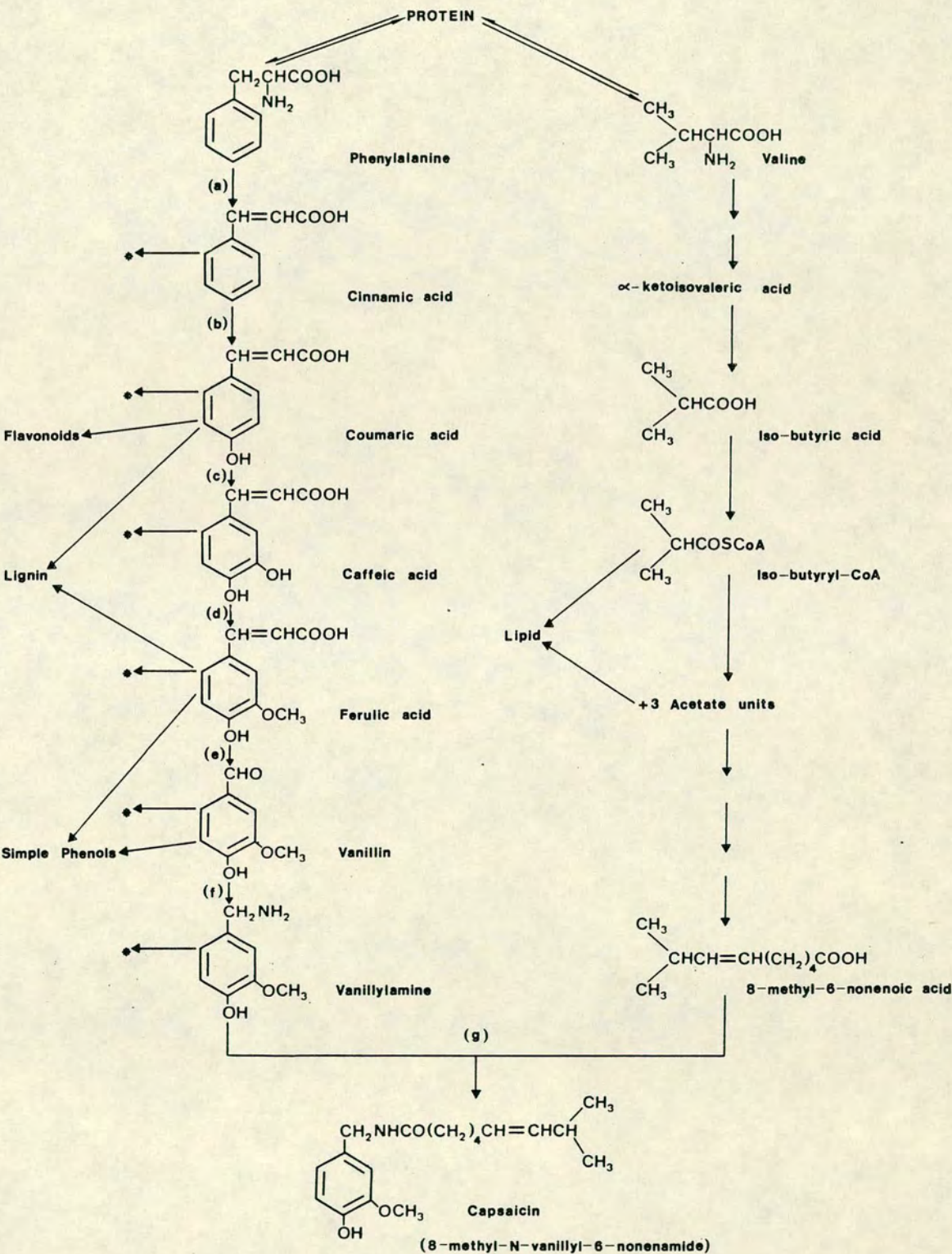
Certain fungal elicitors have been shown to cause dramatic increases in the specific activity of PAL in cultured cells of *C. frutescens* (Holden *et al.*, 1988a,b). In this instance increases in PAL activity were shown to correlate with increased incorporation of [¹⁴C]phenylalanine into capsaicin suggesting that by raising PAL activity, flux through the pathway is intensified and substrate limitation is removed. It would appear therefore that regulation of the capsaicin biosynthetic pathway can be effected in several ways not least of which is availability of substrate supplied to the end of the pathway.

Another interesting feature of the data presented in this thesis is that following an increase in the specific activity of PAL a few hours after subculture, there is a subsequent rise in the specific activity of C,4-H. This suggests that these two enzymes are coordinately induced by a single agent. Indeed there is a substantial amount of evidence available to support the role of coordinate induction of groups of interrelated enzymes (Ebel *et al.*, 1974.; Hahlbrock *et al.*, 1976. and Hahlbrock and Grisebach, 1979). Hrazdina and Wagner (1985) and Wagner and Hrazdina (1984) suggest that phenylpropanoids may be synthesized on membrane associated complexes where the efficiency of substrate channelling is likely to be the overall limiting factor for production of a particular compound and work by Czichi and Kindl (1977) supports this idea. Certainly coordinate induction of interrelated enzymes is a refined way of ensuring that catalytic activities are present when substrates are available and it is likely that some enzymes in the capsaicin biosynthetic sequence are controlled in this way. In addition to the results of this investigation, there is a substantial amount of other evidence supporting the view that capsaicin synthesis is regulated at the end of the pathway. It has been recognized that all the intermediates of the phenylpropanoid pathway from phenylalanine to ferulate are likely to be present in all plant tissues where they are important in cell wall metabolism (Fig. 4.1.). If cell walls from various parts of the chilli plant are subjected to alkaline hydrolysis, all intermediates of the phenylpropanoid pathway as far as vanillin can be detected as the free phenolics (Holden, unpublished results). It is clear therefore, that all reactions as far as vanillin occur in many parts of the plant. However, only the fruit has the capacity to synthesize capsaicin. One interpretation of this fact is that only the fruit has the necessary terminal enzyme activity(ies) essential to complete the reaction sequence to capsaicin.

Fig. 4.1.

The proposed pathway of capsaicin biosynthesis.

where * = esters or glycosides of the acids.



Alternatively, there may be a block in the supply of vanillylamine, 8-methyl-6-nonenoic acid or another substrate further up the pathway as a result of compartmentation or diversion to competing pathways. Certainly it has been shown that a number of phenylpropanoid compounds become incorporated into low molecular weight conjugates and/or the primary cell wall in other systems (Harris and Hartley, 1976; Fry, 1986) and similar results obtained by Holden *et al.* (1987b) showed that a significant proportion of radioactively labelled cinnamic acid "fed" to fruits and cell cultures of *C.frutescens* becomes incorporated into methanol-insoluble material. Intermediates such as those which are removed from the metabolic pool are less available for conversion into capsaicin. It is also likely that a similar situation exists in the acyl branch of the capsaicin pathway where limitation in the supply of iso-type acyl groups might also control the synthesis of capsaicin. This is a possibility since several reviews on plant lipid biochemistry make the observation that the occurrence of branched fatty acids in plants is apparently rare (Hitchcock and Nichols, 1971; Lennarz, 1966; Mazliak, 1973). As "non-equilibrium" enzymes are most likely to provide regulatory points in a biochemical pathway (Newsholme and Start, 1973), the identification of these enzymes is important. A study carried out on immobilized cells of *C.frutescens* (Yeoman *et al.*, 1989) has shown that the pattern of incorporation of the radiolabel from [^{14}C]phenylalanine into intermediates of the pathway was found to be altered in the presence of capsaicin. In particular, there were increases in the levels of incorporation into caffeic acid and ferulic acid and whilst these data by themselves are difficult to interpret, they are at least consistent with the view that a limiting step occurs towards the end of the pathway, possibly the terminal reaction.

In view of the significant amount of evidence supporting the view that a key regulatory step occurs towards the end of the capsaicin biosynthetic pathway, attention was focussed on developing a suitable assay procedure for capsaicin synthase.

Development of an Assay Procedure for Capsaicin Synthase.

The enzymes that comprise the capsaicin synthase complex catalyse the condensation of vanillylamine with iso-type fatty acids to form a range of amides known collectively as the capsaicinoids. Its activity is detectable in fruits and cultured cells of *C.frutescens* only at a particular stage of development. Preliminary studies on this enzyme reaction have been published by a Japanese group who have attempted to assay capsaicin synthase in fruit extracts from *C.annuum* var. Karayatsubusa (a species of chilli pepper similar to *C.frutescens*). From these studies they have proposed a scheme for the biosynthesis of the acyl moieties of capsaicin and its analogues from valine and leucine (Suzuki *et al.*, 1981). Other workers have also demonstrated the incorporation of radiolabelled acyl moieties into capsaicinoids in an *in vitro* system (Kopp and Jurenitsch, 1981) and attempted to assay the enzyme in a cell-free extract from fruits of *C.annuum* (Fujiwake *et al.*, 1980a). These results require careful interpretation because the main technique used for separation of labelled substrate from product (capsaicin) was 1D-TLC. In the present study the resolution for separation of all intermediates of the pathway was found to be inadequate using 1D-TLC. For example, vanillin and several of the capsaicinoids co-chromatograph. The problem can be overcome to a large extent by the use of 2D-TLC and HPLC.

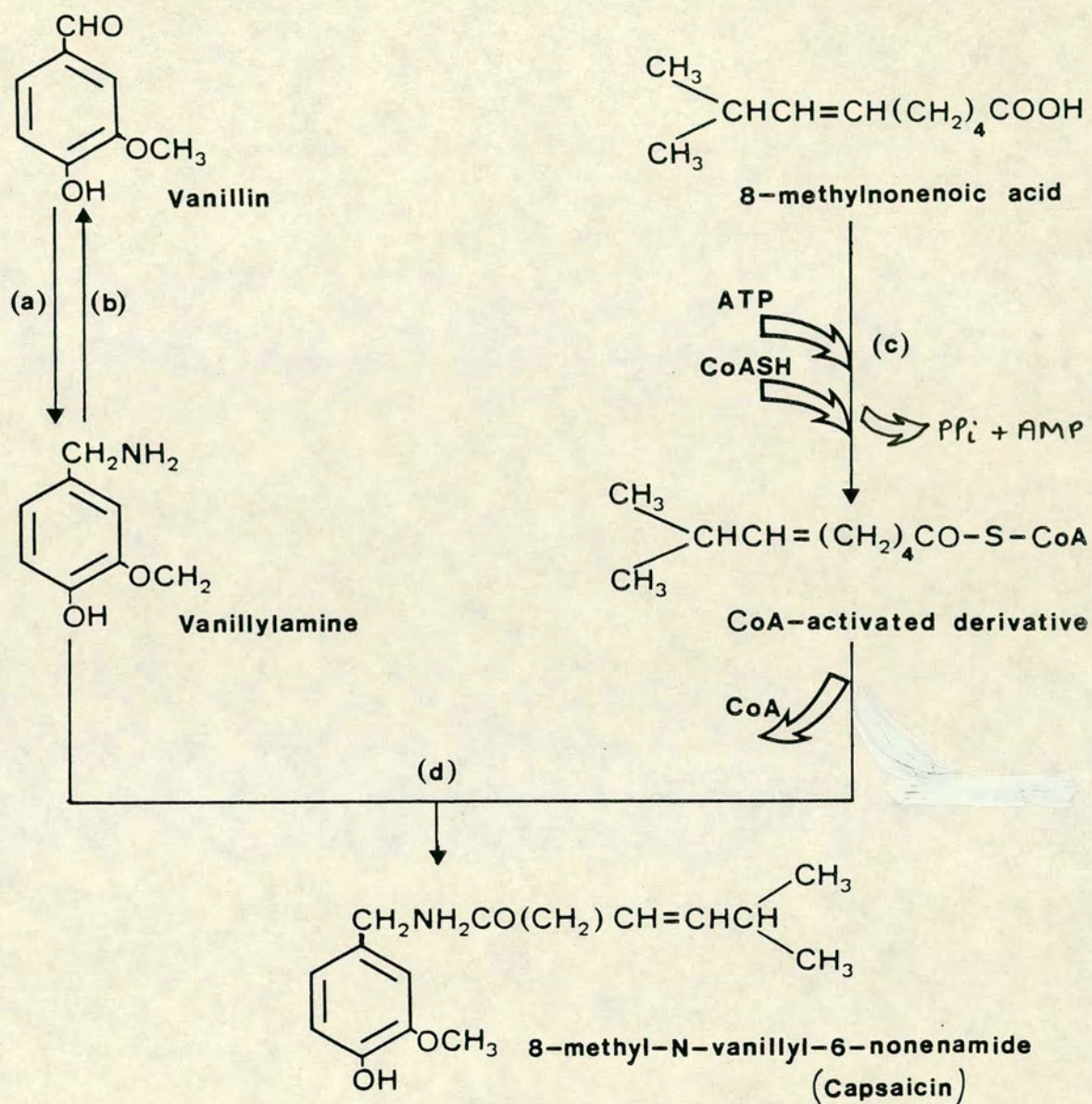
In addition these workers did not attempt to elucidate the mechanism of action of capsaicin synthase or completely determine its localization, although it was suggested that the enzyme might be associated with the tonoplast (Fujiwake *et al.*, 1980b). Furthermore, no attempt was made to correlate capsaicin synthase activity with the onset of capsaicin synthesis in the fruit. Although the methods utilized by the Japanese workers were not as sensitive as the methods employed in this investigation, these results provided a useful starting point in this investigation.

From a careful study of similar reactions reported in the literature it is possible to propose a mechanism for the terminal reaction(s) (see Fig. 4.2.). In this proposed scheme 8-methyl-6-nonenic acid is activated to its CoA derivative in the presence of reduced Coenzyme A and ATP which then condenses with vanillylamine to form the amide 8-methyl-N-vanillyl-6-nonenamide (capsaicin). It is suggested that the

capsaicin synthase complex consists of at least two enzymes, an Acyl CoA-activating enzyme and an Acyl transferase enzyme. Although there is an extensive literature on both of these enzymes in animal systems, the corresponding information on plant enzymes is limited. Thomas *et al.* (1988) showed that an acyl CoA synthetase [E.C. 6.2.1.3] which catalyses the activation of long chain acyl groups was located on the outer membrane of mitochondria extracted from *Pisum sativum*. Interestingly, they also suggested that the location of the acyl transferase which transfers the activated groups [E.C. 2.3.1.21] would appear to be the inner mitochondrial membrane and the possibility of substrate channelling is also mentioned. Barlow and Dixon (1973) demonstrated the presence of choline acetyltransferase in extracts of the nettle *Urtica dioica* and Ichihara *et al.* (1987 and 1988) provided evidence that a number of acyltransferases [E.C. 2.3.1.15.; E.C. 2.3.1.20. and E.C. 2.3.1.51.] are present in maturing safflower seeds. Hares and Frentzen (1987) produced enzymic data on an acyltransferase enzyme obtained from spinach *Spinacia oleracea* leaves and in one report the importance of acyl-CoA synthetase as a pivotal enzyme in acyl-CoA supply was demonstrated (Tomada *et al.*, 1987). In addition, it is suggested that fatty acyl-CoA molecules serve as regulators not only of lipid metabolism, but in other areas of metabolism as well.

Fig. 4.2.

The proposed terminal steps of the capsaicin biosynthetic sequence



(a) Transaminase

(b) Deaminase

(c) Acyl-CoA synthetase

(d) Acyltransferase

(c+d) Capsaicin synthase

In view of the limited information available on methods of assaying these plant enzymes, it was difficult to decide on the best method for assaying capsaicin synthase. A number of techniques for detecting capsaicin were considered; One-dimensional TLC, fluorescence detection and UV spectrophotometry were all rejected on the basis that they were insufficiently sensitive. In addition an HPLC system had already been optimized for the separation and detection of phenylpropanoids and capsaicinoids (see Hall *et al.*, 1987) and other workers have also used HPLC for the quantification of capsaicin (Jurenitsch *et al.*, 1979). However, HPLC would be at the limits of resolution for the quantities of capsaicinoids anticipated in the assay. For these reasons it was decided to attempt to assay capsaicin synthase using the radiolabelled substrate [^3H]vanillylamine. The advantage of such an assay procedure is its sensitivity and the fact that the product could be readily separated from the substrate using HPLC. Ideally the two putative catalytic activities should be assayed independently, and this was initially attempted in a limited number of experiments which were mostly unsuccessful. It was decided that the assay could be developed more quickly using a crude enzyme preparation to which the relevant substrates and cofactors would be added and then necessary refinements made as the development of the assay proceeded. However, some of the experiments which attempted to assay the two enzymes separately produced some interesting results and these are worthy of comment in this discussion.

One such experiment attempted to ensure that the CoA-activated derivative of 8-methyl-6-nonenoic acid was formed during the assay by providing a commercially available acyl-CoA synthetase enzyme in the reaction mixture (Acyl Coenzyme A synthetase [E.C. 6.2.1.3.] from a *Pseudomonas* species (Sigma)). This would then test the ability of the extract to transfer the activated group onto [^3H]vanillylamine and give labelled capsaicin. Although no incorporation of [^3H]vanillylamine into capsaicinoids occurred (Holland, unpublished results), this did not necessarily indicate that the acyl transferase was absent or inactive in the extract since it is highly likely that the acyl CoA synthetase was both species and substrate specific (Kornberg and Pricer, 1953). Certainly the branched chain acyl groups identified in a number of species have been shown to be highly specific and it is unlikely that a CoA synthetase enzyme from a *Pseudomonas* sp. would activate an iso-type acyl group in plants (Hitchcock and Nichols, 1971). Another experiment was carried out using

a number of chemically synthesized CoA-activated derivatives which were added individually to the reaction mixtures along with [^3H]vanillylamine in an attempt to assay acyl transferase activity in the enzyme extract. The results obtained suggested that an acyl transferase enzyme was present in the cell-free extract from chilli pepper fruits and that it had a degree of specificity for iso-type CoA-activated derivatives. It is possible that the substrate specificity of capsaicin synthase for particular acyl groups limits the range of capsaicinoids produced in the fruit, alternatively, it may be that only iso-type acids are supplied down the pathway to capsaicin synthase, as the branch in the pathway which leads to the formation of iso-acyl groups may occur fairly early on as a minor branch of general fatty acid biosynthesis, this would limit the range and quantity of substrates available for use by the enzyme.

Other interesting results were obtained when the two enzymes were assayed together. In particular, it was found that when a crude cell-free extract was assayed for capsaicin synthase activity, virtually no incorporation into capsaicinoids was detected. However, a substantial "back" reaction existed converting [^3H]vanillylamine into vanillin which suggested that capsaicin might be controlling its own formation by negative feedback. These results are in agreement with data obtained by Lindsey (1986) who showed that the addition of capsaicin to immobilized cells synthesizing capsaicin reduced the extent of incorporation of [^{14}C]phenylalanine into the end product. This problem was virtually eliminated in the present investigation by the removal of capsaicinoids from the extract by PD-10 Sephadex chromatography. In another experiment, centrifugal fractionation was used in an attempt to separate the activities of capsaicin synthase and vanillylamine deaminase. The results showed that capsaicin synthase may be associated with a membrane fraction and a number of pieces of evidence would support this view. In particular, Fujiwake *et al.* (1980b and 1982) and Iwai *et al.* (1978) have suggested that capsaicin synthase activity is likely to be associated with the tonoplast, which would seem possible bearing in mind that capsaicinoids are thought to be accumulated in the vacuole (Fujiwake *et al.*, 1980b). Vanillylamine deaminase activity was detected in all the fractions and it seems likely that it is a soluble enzyme associated with the cytoplasm.

Although the assay procedure for capsaicin synthase was sufficient to produce quantities of capsaicinoids detectable by HPLC, it was time consuming. An alternative, more rapid radioactive assay was developed.

Incorporation of [³H]vanillylamine into capsaicinoids *in vivo*.

Capsaicin synthase activity is present in fruits and cultured cells of *C.frutescens* but only at a particular stage of development. The evidence presented in this study demonstrates that [³H]vanillylamine is taken up by cells of chilli fruits and converted into capsaicinoids but only under certain conditions. In particular, it was observed that although the label was taken into cells throughout a time course of fruit development, no detectable incorporation of label into capsaicinoids occurred before day 17 of fruit growth and only after day 18 were high levels of incorporation into capsaicinoids measured.

Similar results were obtained when suspended pepper cells were supplied with [³H]vanillylamine, here it was demonstrated that although the label was taken up into the cells at all ages tested, incorporation of the label into capsaicinoids only occurred in cells approaching the stationary phase. The data suggest that substrate limitation on the phenylpropanoid branch of the pathway is not the only limitation to capsaicin synthesis since vanillylamine was available but not converted. Of course it is possible that compartmentation might reduce the amount of vanillylamine reaching capsaicin synthase and that changes associated with fruit ripening and culture ageing may bring enzyme and substrate into closer proximity. Certainly it is well established that dramatic changes in the constitution of cell walls take place during fruit ripening (Gross *et al.*, 1986; Grierson, 1986; Biale, 1960) and an important part of fruit ripening is cell wall softening in which physical compartmentation is altered. There are also a number of articles in which changes in cell constituents throughout the culture cycle in various species (Amino *et al.*, 1984; Asamizu *et al.*, 1983) have been reported. Although most of these studies concentrate on the growth phase of cell cultures a few have mentioned the changes which might occur during the late growth/early stationary phase and suggest that the biochemical changes which take place during the onset of the stationary phase in cell cultures are likely to be significant. This could result in decreased compartmentation of the cells. Although compartmentation undoubtedly plays an important part in controlling substrate/enzyme interactions early on in development, ultimately capsaicin synthase activity must be present for capsaicin synthesis to occur. In this study it has been shown that capsaicin

synthase activity is only present in the fruits at a particular stage of development and in an extensive experiment where the incorporation of [³H]vanillylamine into capsaicinoids in an *in vitro* assay was measured in fruits of different ages, it was found that label was detected in capsaicinoids as early as day 10 following fruit initiation. This was interesting since incorporation of the label *in vivo* into capsaicinoids did not occur until day 18. The most likely interpretation of these data is that capsaicin synthase is present in the fruit prior to capsaicin accumulation but that substrate limitation controls the onset of capsaicin synthesis. Clearly a number of important changes take place in the fruit prior to capsaicin production because up until day 10 no capsaicin synthase activity is detectable. The activity develops between day 10 and 17 but no capsaicin is synthesized until another key change takes place in the fruit at *ca.* day 18, this may be the sudden availability of certain substrates and more specifically, the availability of suitable acyl-CoA derivatives. Thus as for all enzymically controlled steps, product formation can be regulated by availability of substrate as well as enzyme activity.

The Regulation of Capsaicin Biosynthesis

The onset of capsaicin synthesis is accompanied by significant changes in the metabolic activity of fruits and cultured cells of *C.frutescens* (Hall *et al.*, 1987) which presumably occur as a result of alterations in enzyme activities and substrate availability. Capsaicin accumulation is associated with fruit ripening and this process has been investigated by a number of research groups who have attempted to pin-point the key biochemical event which triggers the ripening process. Gross *et al.*, (1986) demonstrated that fruits of *C.annuum* underwent a respiratory climacteric during fruit ripening which is believed to be associated with an increase in ethylene evolution immediately prior to the rise in respiration rate (McGlasson, 1985). Although they showed a significant rise in the respiration rate, no increase in the levels of ethylene produced were reported in *C.annuum*. Many other reports correlate ethylene production with the onset of ripening (Sims *et al.*, 1970; Lee *et al.*, 1973; Engelsma and Van Bruggen, 1971) and treatment with various ethylene evolving agents eg. Ethrel (Sims *et al.*, 1970) and Ethephon (Lee *et al.*, 1975) have been shown to hasten ripening in chilli pepper fruits. Other results obtained by Lee *et al.*, (1975) showed that in response to Ethephon treatment, the oxygen concentration in the fruit interior decreased. This is particularly interesting bearing in mind that reducing conditions would tend to increase capsaicin synthase activity and limit the rate of the vanillylamine deaminase reaction.

Gross *et al.*, (1986) noted that ripening of chilli peppers was accompanied by a loss of certain carbohydrates from the cell wall, in particular arabinose and galactose which were released in large amounts. In addition a large increase in the activity of β -galactosidase was measured as fruits ripened and developed their red colour. Changes which take place in the cell wall during fruit ripening could provide large quantities of substrates for capsaicin biosynthesis. In addition, it has been shown that some cell wall fragments may display biological activity (Albersheim and Darvill, 1985) and it is possible that release of such compounds from the cell wall might elicit changes leading to the onset of capsaicin biosynthesis for example, enzyme induction. In fact, a fungal cell wall preparation has been shown to increase capsaicin biosynthesis in cultures of *C.frutescens* (Holden *et al.*, 1988a).

A large amount of work has been carried out on the biosynthesis of

secondary products in the cultured cell system. It has been shown by a number of workers that most rapidly growing cultures do not accumulate secondary products to any great extent because precursors of the desired product are required in primary metabolic processes essential for cell growth and division (Hall and Yeoman, 1986). Indeed Lindsey and Yeoman, (1983) showed that an inverse correlation exists between growth phase and alkaloid content of cells of *Datura innoxia*, and Cvikrova *et al.*, (1988) demonstrated considerable increases in the levels of phenolic substances during the early stationary phase of suspended cells of *Nicotiana tabacum*. In the literature there is a substantial amount of evidence, in different species, to support this view (Ramawat and Arya, 1979; Westcott and Henshaw, 1976; Nash and Davies, 1972; Forrest, 1969). Accordingly, it has been suggested that the transition from primary metabolism to secondary metabolism is the result of a change in enzyme activities and that certain enzymes may be induced/activated with the onset of stationary phase. It has also been shown that cultured cells grown under conditions of reduced growth and subjected to selected stress treatments showed substantial increases in extractable capsaicin synthase activity during the down-turn of growth (*ca.* 21d. following subculture). In addition, in a number of cases an additive effect of the treatments was observed where capsaicin synthase activity would be increased substantially more by two treatments than by one. For example, elicitation and minus nitrogen treatments applied simultaneously increased the amount of extractable capsaicin synthase activity more than either treatment alone. This suggested that the changes in metabolism which occur associated with reduced growth of cell cultures can increase the activity of capsaicin synthase directly. Furthermore, the pattern of incorporation of [^{14}C]phenylalanine into products differed in nutrient stressed cells and control cultures indicating that the flow of metabolites through the capsaicin pathway was altered, possibly as a result of changes in the activities of particular enzymes. All of these data support the view that capsaicin synthesis is controlled by the activities of particular enzymes and the availability of substrates to those enzymes.

Possible Regulation of Enzyme Activities.

The evidence provided in this investigation shows that capsaicin synthase activity is only present at a particular stage of development associated with the onset of ripening in chilli pepper fruits and with the stationary phase in cultured cells. Since the activity is not present at all times, it is likely that the enzyme is induced/activated by one or more agents when ripening and the down-turn of growth occur in fruits and cultured cells respectively. There are several levels at which the production of proteins are regulated; one is transcriptional control, the regulation of the transcription of DNA to mRNA coding for a given protein or set of proteins, and another is translational control, the regulation of the initiation and rate of synthesis of polypeptide chains. Certainly it is now generally accepted that the ripening process involves the expression of developmentally regulated genes (Grierson *et al.*, 1981a; Grierson, 1983 and 1986). It is also recognized that fine control of metabolism can be effected by various post-transcriptional events including processing and transport of mRNA, post-translational modification of the protein and changes in compartmentation of enzyme and substrate. For example, Budde and Chollet (1988) have demonstrated the regulation of enzyme activity in plants by reversible phosphorylation.

It has been mentioned already that dramatic changes in the cell walls of fruits take place during ripening and that release of certain fragments from the cell wall might elicit changes in enzyme activities leading to the onset of capsaicin synthesis. There is a substantial amount of evidence supporting the view that large increases in the activities of some enzymes occur during the ripening of many fruits. It has also been shown that ripening fruits retain the capacity to synthesize RNA and proteins (Grierson, 1983) and work by Hulme (1954) indicated that there is a net increase in protein content during the ripening of apples. Gross *et al.*, (1986) measured a 50-fold increase in the activity of β -galactosidase from the immature green to the red ripe stage of chilli pepper fruits. This enzyme can hydrolyse lactose to its components, glucose and galactose, it is also the classic example of an inducible enzyme in bacteria which is induced directly and rapidly by the substrate lactose. Another enzyme associated with fruit ripening is polygalacturonase which is responsible for cell-wall degradation. Hobson (1964) demonstrated that this enzyme activity appears during tomato ripening and that there is a correlation between fruit firmness and enzyme activity (Hobson, 1965). Other work by Grierson and

Tucker (1983) showed that enhanced ethylene biosynthesis occurs prior to polygalacturonase production and that ethylene triggers enzyme synthesis indirectly. In addition, it has also been suggested that ethylene production results in a rise in the activities of certain enzymes associated with fruit ripening; Engelsma and Van Bruggen (1971) studied the relationship between ethylene synthesis and enzyme induction in plant tissue. They found a correlation between ethylene evolution and PAL activity in excised Gherkin tissue and suggested that ethylene was acting as an enzyme inducing agent. The presence of isozymes of polygalacturonase has invited the suggestion that different forms of the enzyme may be induced in response to various factors (Pressey and Avants, 1973), particularly as isozymes provide a potential control over pathways which may be active under some conditions but not under others. Another cell-wall solubilizing enzyme, cellulase, has been shown to be synthesized during avocado ripening (Tucker and Laties, 1984; Christoffersen *et al.*, 1984) and an increase in the activity of acid invertase has been demonstrated during the ripening of tomatoes (Grierson *et al.*, 1981b).

A number of other reports have shown the presence of inducible enzymes which are active at the late growth/early stationary phase of cell cultures; Vornam *et al.*, (1988) measured increases in the activity of stilbene synthase in cultured peanut cells 12–16h. after transfer to fresh medium. In addition, they observed a second higher peak of stilbene synthase activity at the end of the growth cycle. Other data showing large rises in PAL and in some cases *p*-coumarate:CoA ligase in stationary phase cell cultures were provided by Hahlbrock *et al.*, 1971; Ebel *et al.*, 1974); Westcott and Henshaw, (1976). Cvikrova *et al.*, (1988) observed dramatic increases in the activity of PAL immediately prior to the accumulation of phenolics at stationary phase in *Nicotiana tabacum*. These results all suggest that enzyme induction plays an important part in the regulation of secondary product formation.

In view of the data and evidence provided, it is probable that capsaicin synthase is activated/induced at a precise stage in the development of fruits and cell cultures of *C.frutescens*.

Availability of Substrates.

So far, regulation of enzyme activities has been discussed in relation to the regulation of capsaicin synthesis and accumulation. However, the availability of substrates upon which the enzymes act has also been shown to be important in regulating product synthesis (Margna, 1977).

It was shown that in fruits of *C. frutescens* [^3H]vanillylamine was taken into cells throughout a time course of fruit development and yet incorporation into capsaicin only occurred *ca.* 18d. following fruit initiation. In addition, the *in vitro* assay of capsaicin synthase throughout the same time course demonstrated that capsaicin synthase activity was present as early as day 10 and yet no incorporation *in vivo* into capsaicinoids was detectable. A similar observation was made in suspended cells supplied with [^3H]vanillylamine *in vivo* in which uptake occurred in all cell ages tested. However, incorporation into capsaicinoids was limited to cells approaching the stationary phase. These data are consistent with the view that substrate availability may play an important role in the regulation of capsaicin biosynthesis, and that changes in substrate availability appear to be correlated with the age/developmental stage of the cells.

The availability of substrate and how it may be controlled will now be considered.

1) Changes in Metabolism.

The changes associated with reduced plant cell growth in both fruit and cultured cells are complex and the production of at least some types of secondary metabolites appears to increase as the growth rate is reduced. There seems to be a "switch" in the biochemistry of plant cells at a particular stage of development resulting in a diversion from primary to secondary metabolism (Yeoman *et al.*, 1980). Reduced primary metabolism and cell growth tend to increase the availability of certain substrates for secondary metabolism. Indeed many reports have demonstrated that under conditions of reduced culture growth there is a corresponding increase in the production of one or more secondary metabolites (Lindsey, 1985; Lindsey and Yeoman, 1983; Ravishankar *et al.*, 1988). The data presented here also show that under conditions of reduced growth, increases in the incorporation of [^{14}C]phenylalanine into capsaicin are detectable, possibly as a result of the increased availability of

phenylalanine, valine or both precursors. Also in the fruit, production of secondary metabolites associated with the ripening process occurs at a time of reduced growth and a down-turn in primary metabolism.

It has been suggested that the regulation of the ripening process in fruits is governed by the respiration rate (Biale, 1960) and that ultimately the energy charge of cells dictates which pathways of metabolism will be operational. Atkinson (1977), has suggested that the energy charge is a major factor in the regulation of pathways that produce and utilize high-energy phosphate groups and that if the ATP/AMP ratio is high, the system is "filled" with high energy bonds and the biosynthetic capacity is great. However, if all the adenine nucleotide is in the form of AMP, the energy charge is zero and the biosynthetic capacity low. Clearly at any given time within a cell, catabolic and anabolic processes must be operating simultaneously. It is therefore necessary for the cell to separate enzymes, substrates and entire metabolic sequences in some cases so that all essential processes can occur without the useless dissipation of energy and carbon skeletons. This introduces the idea of compartmentation which undoubtedly plays a key role in the regulation of secondary metabolism in general and capsaicin biosynthesis in particular.

2) Compartmentation.

There is a significant amount of published work which supports the view that separate pools of metabolites and enzymes exist as a result of compartmentation (Allen *et al.*, 1976; Da Cunha, 1987; Oaks and Bidwell, 1970; Sols and Marco, 1970). Intracellular compartmentation of enzymes and enzyme systems ensures that metabolic sequences which might have common substrates are separated and controlled independently. Compartmentation can be divided into two main categories; physical compartmentation, where substrate/enzyme interactions are limited due to the presence of a physical barrier separating one from the other and chemical compartmentation where substrate may be limited because it is bound to another molecule and thus not "free" to be a substrate for an enzyme. Both of these mechanisms are likely to play a role in the regulation of secondary metabolism and a number of reports have emphasized how important compartmentation of substrates is in the control of secondary product formation and accumulation. Changes in the physical compartmentation properties of cell walls at the onset of ripening have already been mentioned (Gross *et al.*, 1986). Another report by Legge *et al.*

, (1986) has shown that changes in the molecular organization of membranes in pericarp cells of tomato occur as a result of the ripening process. They have suggested that the increased fluidity of the membrane enhances the permeability properties of the membrane and allows more movement of substrates. In addition, Biale (1960) has shown that disruption of mitochondrial membranes occurs during fruit ripening and that the disruption is correlated with reduced ATP production and a change in the energy charge of the cells. Other work has shown that chemical compartmentation can play a role in the removal of potential substrates from the metabolic pool; in particular, a number of reports have shown that the cell wall is a major site of accumulation of phenolic compounds and that glycosides and esters of phenolic acids effectively represent a reservoir of substrates which are used in other secondary metabolic pathways (Hrazdina and Wagner, 1985; Fry, 1983 and 1984; Holden *et al.*, 1987b). During down-turn of cell growth, it is suggested that breakdown of these compartments may result in release of substrates. Clearly biochemical events occur prior to the breakdown of such compartments and the onset of secondary metabolism must be signalled early on in fruit development before gross structural changes take place. The nature of early signals is not clear but Ca^{2+} and calmodulin have been implicated in control of plant metabolism (Marme, 1985; Gilroy *et al.*, 1987) and it is possible that they may be involved in the regulation of capsaicin synthesis.

In attempting to summarize the possible regulation of capsaicin biosynthesis, it is important to stress the fundamental changes which take place upon the down-turn of cell growth. It may be that changes in the ATP/AMP ratio within a particular compartment result in the formation of secondary messengers which activate enzymes associated with the breakdown of cell walls, leading to the release of certain intermediates of the phenylpropanoid and/or acyl branches of the pathway. In addition, the early changes in cell biochemistry which occur upon down-turn of growth might also induce/activate selected enzymes associated particularly with capsaicin biosynthesis. The availability of substrates coupled with the presence of selected active enzymes would facilitate the synthesis and accumulation of capsaicin.

Future Work

The results obtained in this investigation open up many avenues for future research into the regulation of capsaicin synthesis in *C.frutescens*. Here, objectives for future work are outlined.

In the short term:

1) Determination of the specific activity of capsaicin synthase in cultures under inductive conditions which produce and under non-inductive conditions which do not produce capsaicin. It will be important to screen high yielding cell lines of *C.frutescens* for a) their ability to produce capsaicin and b) capsaicin synthase activity. Through this study the presence or absence of a correlation between enzyme activities and capsaicin synthesis could be established.

2) Should a correlation exist between capsaicin synthase activity and capsaicin synthesis in cultures of *C.frutescens* (consistent with the evidence presented here), it would then be necessary to confirm the number of reactions catalysed by capsaicin synthase. This may be done by assaying the enzymes *in vitro* in the presence of capsaicin which might be expected to eliminate capsaicin formation through negative feedback and allow accumulation of intermediary forms of 8-methyl-6-nonenoic acid. It is also possible, to chemically synthesize acyl-CoA derivatives (Seubert, 1960) from the acid chloride of the fatty acid. This would then make the separate assay of both activities possible. Kinetic analysis of the unpurified enzymes would demonstrate cofactor/modulator requirements and give an indication of which reaction is controlling the overall rate through the complex under defined conditions. The limiting protein would then be targetted for purification.

3) It is recognized that there are large problems associated with the isolation and purification of enzymes, specifically those associated with secondary metabolism, particularly in view of the fact that the enzyme of interest may only account for <0.1% of the total protein extracted (Berlin *et al.*, 1985). Purification of the protein would involve a range of techniques including; ammonium sulphate precipitation, size exclusion chromatography, affinity chromatography and electrophoresis. During the purification procedure extensive kinetic analysis of the enzyme would provide further information on the nature of cofactor requirements and would suggest possible mechanisms

for the control of capsaicin synthesis which could be tested *in vitro*.

In the long term:

It would be possible to determine whether or not capsaicin synthase activity was regulated at the level of gene transcription, mRNA translation, transport or modification or at the level of post-translational modification.

The investigations described above will lead to a greater understanding of the regulation of capsaicin synthesis in *C.frutescens* and primary and secondary metabolism in general. At present the field of secondary metabolism offers great potential, particularly in the area of molecular biology since genetic manipulation of secondary metabolic sequences is not so likely to result in production of lethal genotypes as is manipulation of primary metabolic processes.

Appendices

Appendix A.

The experiments in this appendix were designed to study and analyse the growth kinetics of suspended cells of *C.frutescens* grown on standard SH medium (section 2.2.1.1.). Several parameters were measured at selected time intervals throughout the culture cycle:

- fresh weight
- dry weight
- total cell number
- % cell viability
- pH of media
- soluble protein content

Suspended cells of *C.frutescens* (14 day old) which had been maintained under normal culture regime (section 2.2.3.4) and growth conditions (section 2.2.3.1) for 3 successive subcultures were subcultured from one stock flask into 250ml Erlenmeyer flasks containing fresh medium ($2.0\text{g} \pm 0.08\text{g}$ per flask). The cells were grown under normal growth conditions for the duration of the experiment. At selected time intervals following subculture, 3 replicate flasks were taken, the cells harvested (section 2.3.) and growth parameters were determined as follows:

Fresh weight	section 2.3.1.
Dry weight	section 2.3.2.
Total cell number	section 2.3.3.
% cell viability	section 2.3.4.
pH of media	
Soluble protein content	section 2.5.2.3.

Fig.A(1).

The changes in fresh weight which take place subsequent to the subculture of a cell suspension culture of *C.frutescens*.

Fig.A(2.)

The changes in dry weight which take place subsequent to the subculture of a cell suspension culture of *C.frutescens*.

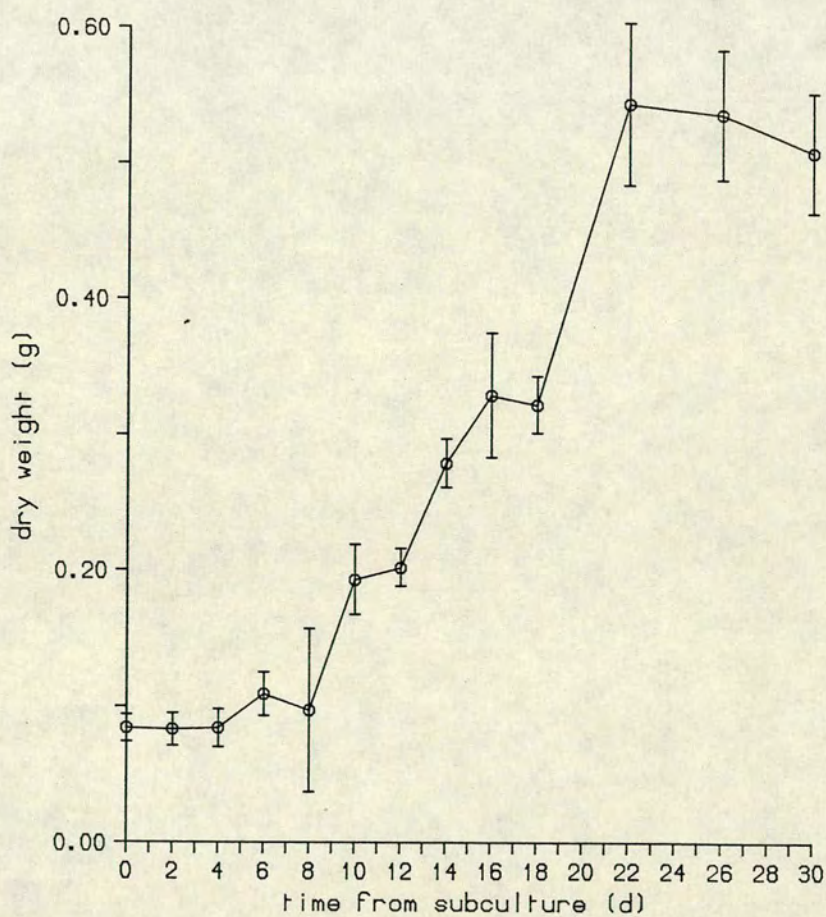
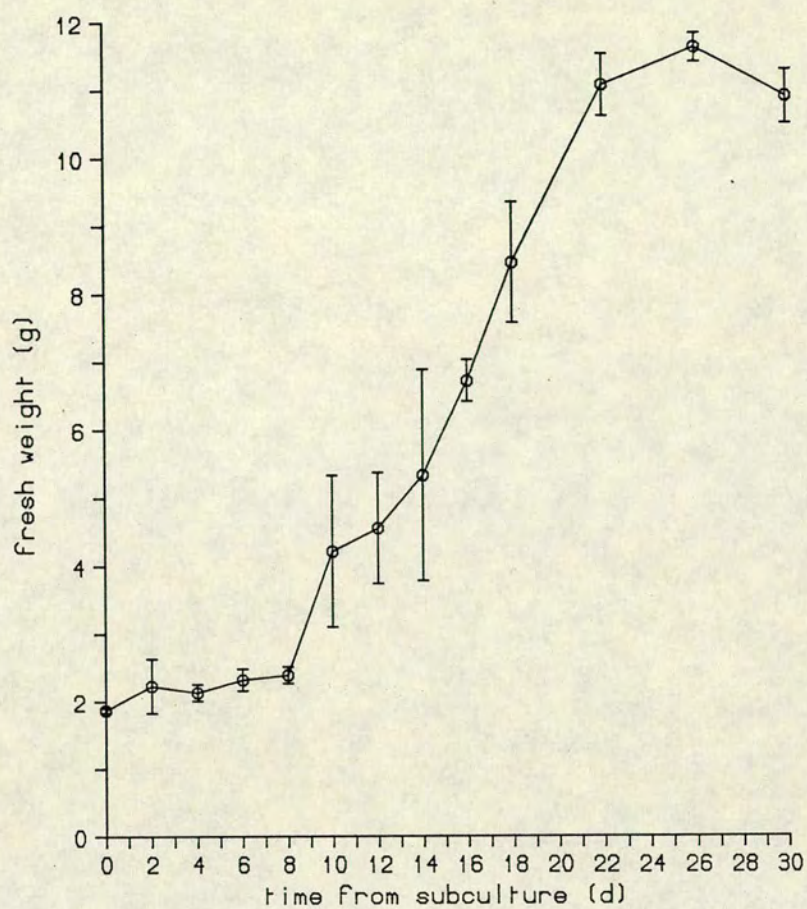


Fig.A(3).

The changes in cell number which take place subsequent to the subculture of a cell suspension culture of *C.frutescens*.

Fig.A(4).

The changes in cell viability which take place subsequent to the subculture of a cell suspension culture of *C.frutescens*.

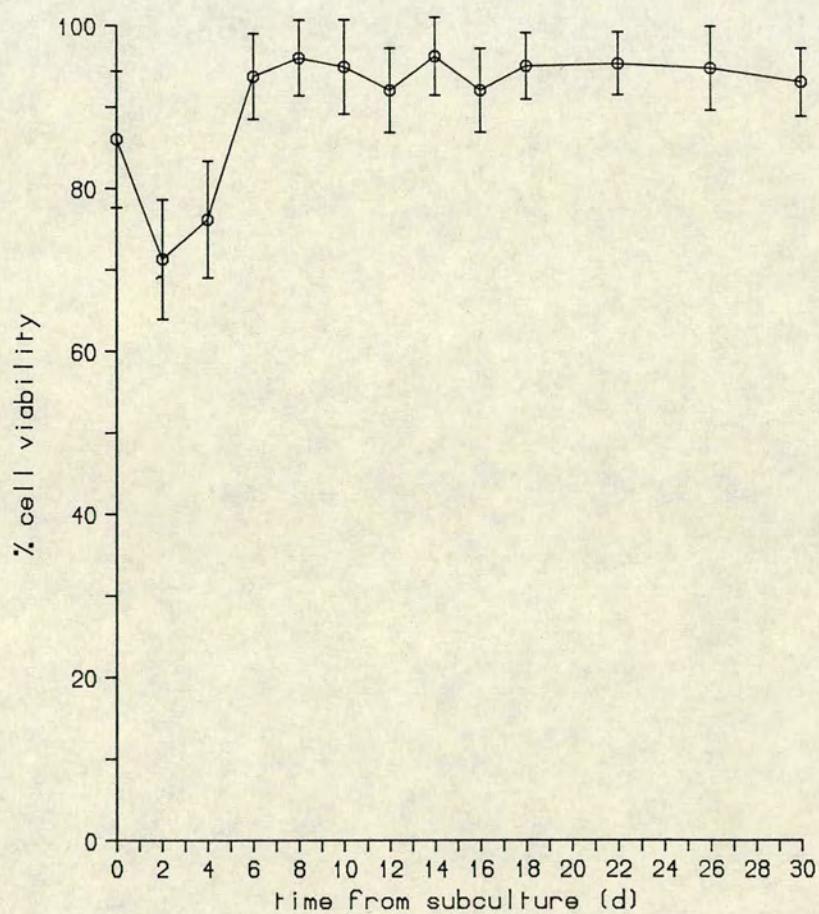
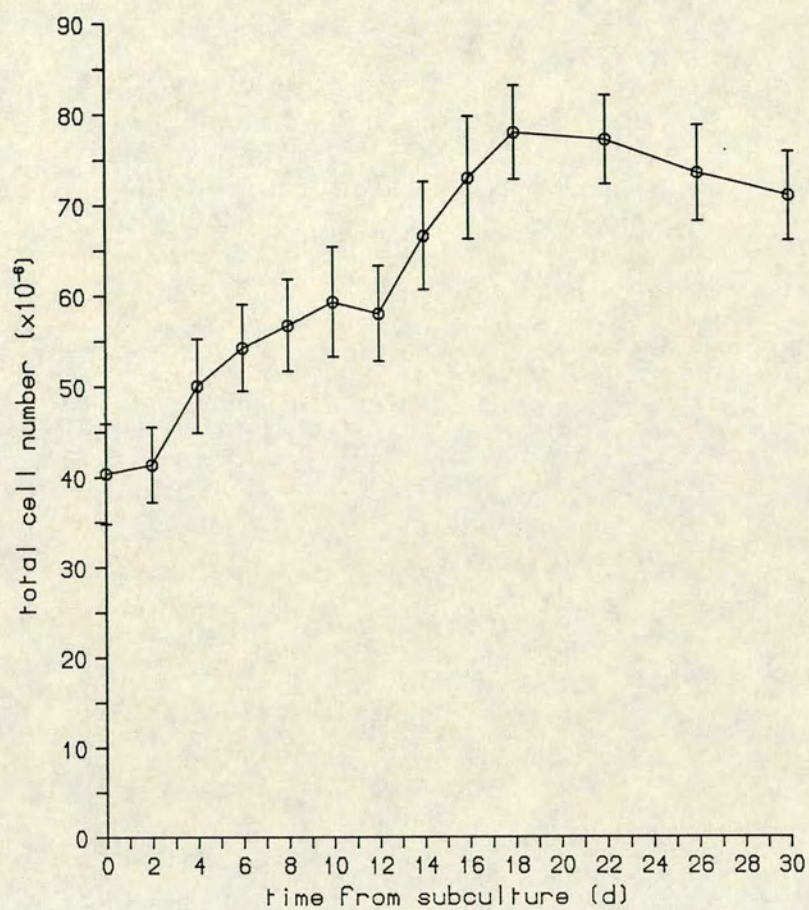
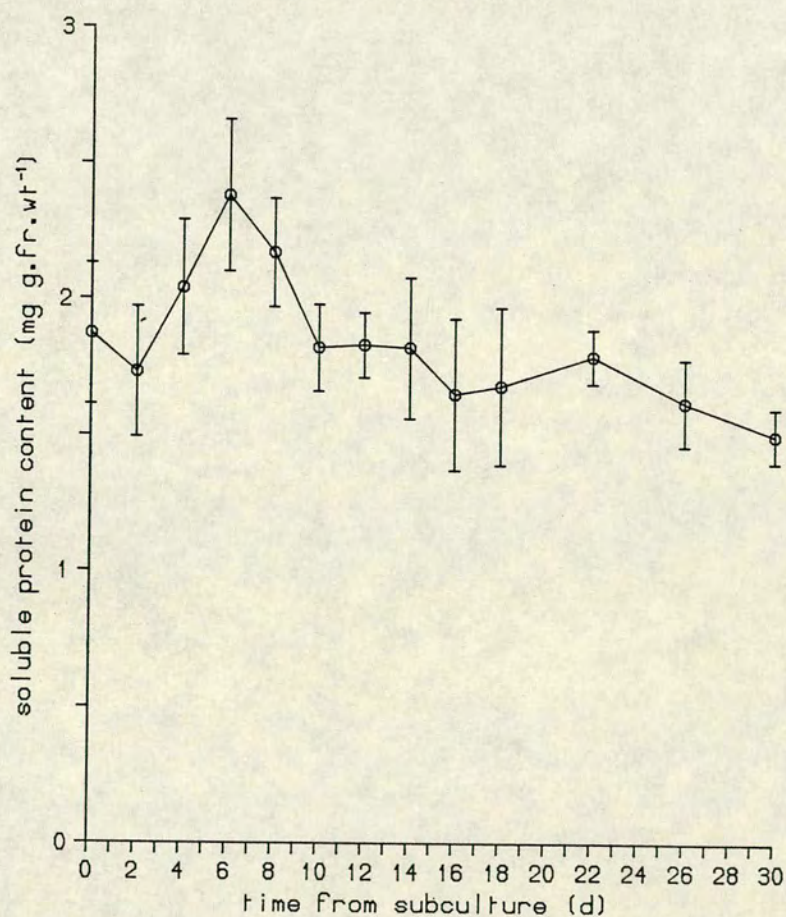
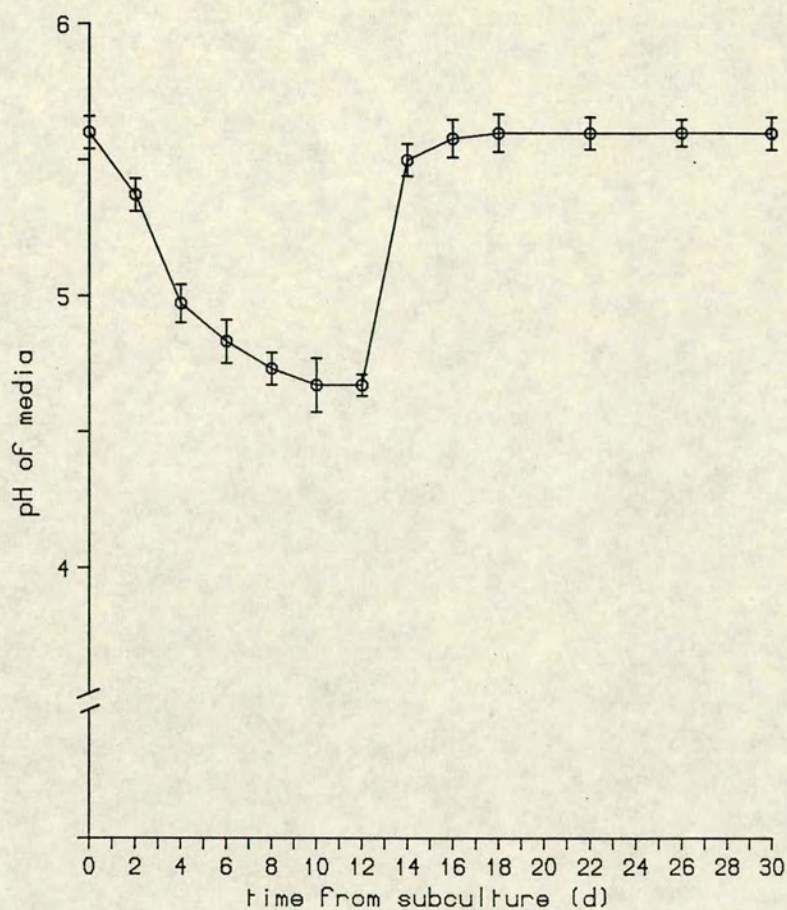


Fig.A(5).

The changes in pH of the medium which take place subsequent to the subculture of a cell suspension culture of *C.frutescens*.

Fig.A(6.)

The changes in soluble protein content of the cells which take place subsequent to the subculture of a cell suspension culture of *C.frutescens*.



The results presented in Fig.A(1). show the changes in fresh weight throughout a culture cycle of *C.frutescens*. A temporary lag phase was shown to precede the increase in fresh weight which increased steadily from day 8 to *ca.* day 22 after which time there was a levelling off.

The pattern of dry weight increase (Fig.A(2).) in these cultures was similar to that for fresh weight. Maximum levels were reached by day 22.

After an initial brief lag-phase of *ca.* 5 days (Fig.A(3).), the total cell number of these cultures increased until day 18. After day 18, the number of cells per culture remained relatively constant.

With the exception of a temporary decline immediately following subculture cell viability was very high in these cultures. A maximum of 97% was found at day 14 (Fig.A(4).).

In Fig.A(5). the changes in the pH of the medium are shown. The pH dropped steadily by *ca.* 1 pH unit (from 5.6 to 4.6) over the first 6 days following subculture. The low pH was maintained for a further 6 days after which time it increased to 5.6 by *ca.* day 16.

The soluble protein content of the cells throughout a culture cycle is shown in Fig.A(6). There was a fairly rapid increase in the level of extractable soluble protein over the first 6 days (from 1.8 – 2.5 mg. g.f.wt.⁻¹) after which time the level returned to *ca.* 1.8 mg g.f.wt.⁻¹ and remained at that level for 12 days. In the later stages of the growth cycle (days 22–30), there was a small but significant decline in the soluble protein content of the cells.

Appendix B.

B(1).

Measurement of the effect of enzyme concentration on the velocity of the capsaicin synthase reaction.

Under given conditions, two molecules of enzyme acting independently in solution will convert twice as much substrate in a given time as one molecule; so if a reaction is catalysed by an enzyme, the rate of the reaction should be proportional to the concentration of enzyme. This experiment was designed to confirm that a linear relationship between rate and enzyme concentration exists. Departures from linearity can be due to a number of factors:

1. the presence of toxic impurities in the enzyme preparation.
2. the presence of a dissociable activator in the enzyme preparation.
3. a limitation / artefact of the assay procedure.

Information obtained in this type of experiment not only serves to demonstrate the linear relationship between rate of reaction and enzyme concentration, but can also give important information if the relationship deviates from linearity.

Suspended cells of *C.frutescens* grown on MS media were subcultured and subjected to treatments as for culture (MS)S(H) in experiment 3.6. to induce capsaicin synthase activity. Cells were harvested at T=36 d. (for time-scale see Fig. 3.6a.) and extracted for capsaicin synthase activity (section 3.6.). The enzyme extract was assayed for capsaicin synthase activity (section 2.5.2.2 [system 2]) over a range of protein concentrations. The reaction mixtures were extracted in chloroform (section 2.6.1.2) and the samples analysed as for section 3.6.

The data presented in Fig.B(1). show the relationship between protein (enzyme) concentration and capsaicin synthase activity (dpm. h.^{-1}). It can be seen that a linear relationship exists between the two parameters over the protein concentration used. Regression analysis of the data showed the relationship to be linear and a correlation coefficient of 0.98 was obtained. Since the relationship between enzyme concentration and rate of reaction is linear, further kinetics were measured.

Fig.B(1).

The effect of protein (enzyme) concentration on the rate of the capsaicin synthase reaction.

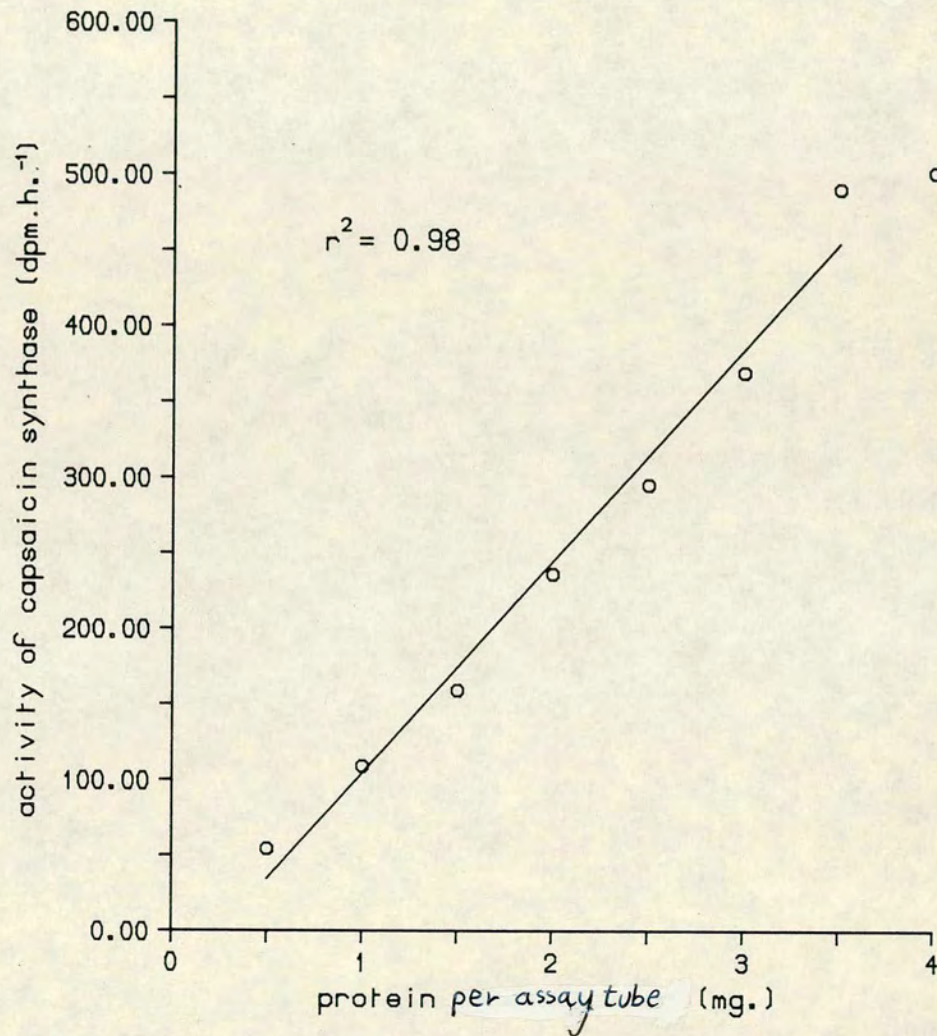
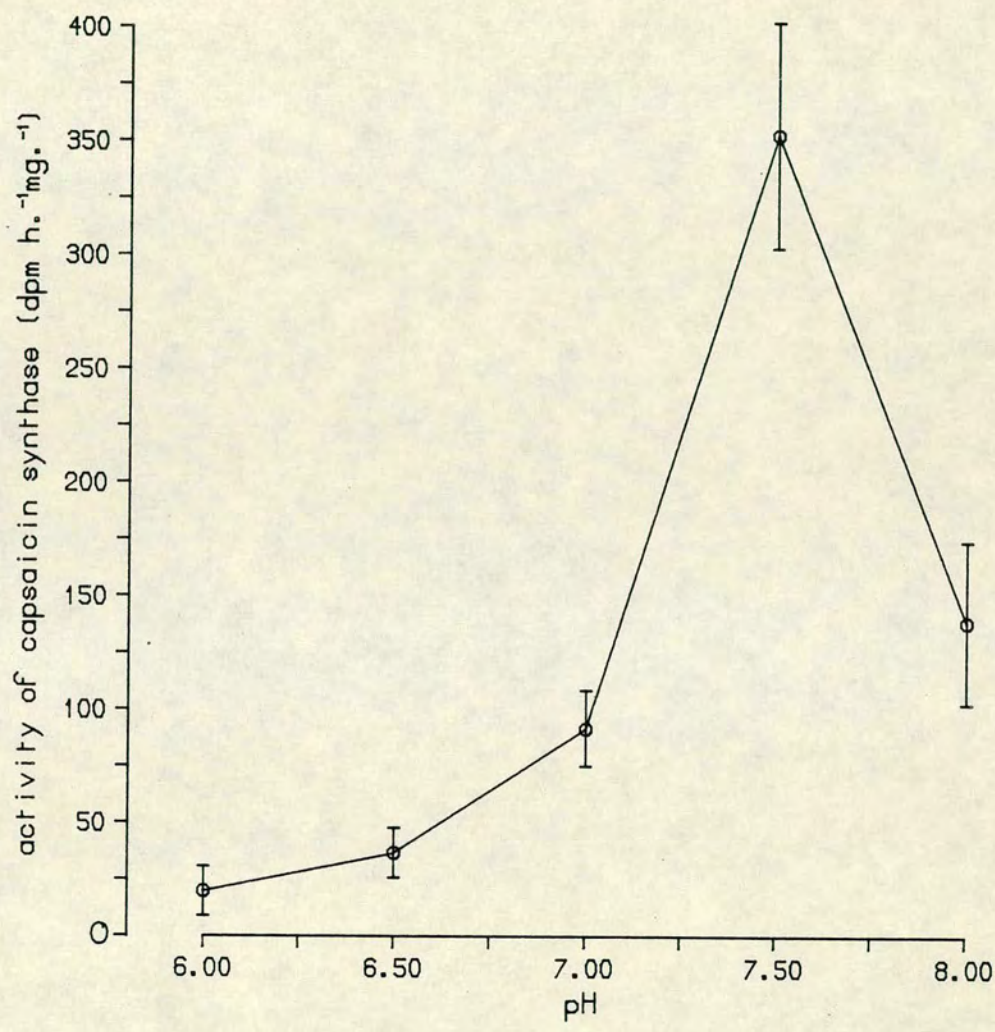


Fig.B(2).

The relationship between activity of capsaicin synthase and pH.



The results presented in appendix B(1). show that the relationship between enzyme concentration and reaction rate is linear over the concentration range tested. Experiments in this section now concentrate on measuring certain kinetic parameters of the capsaicin synthase reaction(s).

B(2).

Determining the effect of pH on the activity of capsaicin synthase extracted from cultured cells of *C.frutescens*.

In general, enzymes are only active over a limited range of pH and in most cases a definite pH optimum is observed (see section 3.3.5.). This experiment was designed to determine the pH optimum for capsaicin synthase extracted from cultured cells of *C.frutescens* so that the enzyme could be assayed at the optimal pH in future experiments. The pH optimum in fruits of *C.frutescens* was found to be *ca.* 7.4, so a pH range was chosen in this experiment with this optimal value in mind.

Capsaicin synthase was extracted and assayed as described in appendix B(1). The buffer range was prepared as in section 3.3.5. Subsequent analysis of the samples was carried out as for appendix B(1).

The data presented in Fig. B(2). show the relationship between pH and capsaicin synthase activity. The enzyme is active over a fairly narrow pH range and a pH optimum of *ca.* 7.5 was observed. These data are consistent with results obtained from the fruit enzyme.

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